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(54) Title: CELLULAR REGULATORS OF INFECTIOUS AGENTS AND METHODS OF USE

(57) Abstract: The invention provides ribozymes with target recognition sequences that allow the ribozyme to target and cleave cellular regulators that are involved in HCV and other viral replication. Also provided are nucleic acids encoding various cellular regulators and sequences in such nucleic acid for which ribozymes can be directed. Fragments of these nucleic acid and protein sequences also are provided. Further provided is a method for identifying a ribozyme reactive with a cellular regulator of viral replication or expression, and a method for identifying the cellular regulator targeted by such ribozymes. Also provided is a method of identifying a compound that modulates the activity of a cellular regulator. Further provided is a method of treating an HCV infection.

## CELLULAR REGULATORS OF INFECTIOUS AGENTS AND METHODS OF USE

### BACKGROUND OF THE INVENTION

5           This invention relates generally to infectious diseases, and more specifically to methods of identifying cellular regulators essential in the pathogenesis of infectious agents.

          Infectious diseases are a serious cause of death and debilitation in the United States and particularly in the non-industrialized populations of world. In particular,  
10   Hepatitis C virus (HCV) is the major cause of non-A, non-B hepatitis, which is also a serious worldwide health problem. Approximately 1% to 2% of the world population is infected with HCV. In the United States alone, there are approximately 2.7 million infected individuals, with 150,000 acute cases occurring annually, resulting in HCV infection as the ninth leading cause of death.

15           Intravenous drug abuse has been indicated as one important risk factor for transmission of HCV. However, different epidemiological studies have revealed that for up to 20 to 40% of patients chronically infected with HCV, no known risk factors have been identified.

          The disease associated with HCV is, in most cases, benign. Nevertheless,  
20   persistent infection can lead to liver cirrhosis and hepatocellular carcinoma. HCV disease can be manifested as acute viral hepatitis which is usually clinically mild, but may develop into a severe or fulminant hepatitis. Chronic HCV hepatitis is believed to occur more frequently than hepatitis B virus, especially following post-transfusional acute hepatitis C disease.

25           Treatment of HCV infection has primarily been with alpha-interferon. In some instances liver transplantation has been performed for end-stage hepatic deficiency, but invariably the transplanted liver also becomes infected with HCV and ultimately fails.

          Virally encoded gene products have been thought to be effective targets for drug development because they are unique to infected cells. However, despite the potential  
30   specificity of drugs targeting viral gene products, they have the disadvantage of rapidly becoming ineffective due to the ability of the virus to mutate and become drug resistant. This drug resistant phenomenon has been observed with both DNA and RNA virus.

Moreover, similar phenomenon have been observed with other infectious agents such as paracytes which change coat proteins in response to specific targeting agents or host immune responses. In contrast, cellular genes that are essential for viral replication or expression are not rapidly mutated and therefore less susceptible to developing  
5 resistance. The availability of such cellular genes would be valuable as targets for development of new therapeutics and methods for treatment of a variety of viral and other infectious diseases.

Thus, there exists a need for the rapid and efficient identification of cellular genes involved in the propagation or pathogenesis of infectious agents.

10 The present invention satisfies this need and provides related advantages as well.

#### SUMMARY OF THE INVENTION

The invention is directed to methods of identifying cellular regulatory genes that support the replication of viruses such as HCV. The methods are directed to the  
15 identification of ribozymes that target such cellular regulatory genes and to identifying the genes targeted by the ribozymes.

Accordingly, the present invention provides ribozymes having target recognition sequences that enable the ribozymes to bind and cleave cellular regulators involved in viral replication including HCV virus. Such sequences encode a variety of ribozymes  
20 and their associated target recognition sequences as well as the cellular regulators.

Also provided is a method of identifying a compound that modulates the activity of a cellular regulator. The method consists of contacting a sample containing a cellular regulator and a nucleic acid element acted on by a cellular regulator with a test  
25 compound under conditions that allow replication or expression of the nucleic acid element or a gene operatively linked to the nucleic acid element, and measuring the amount of replication or expression of the nucleic acid element or gene, an increase or decrease in the amount of replication or expression in the presence of the test compound compared to the absence of the test compound indicates that the compound has cellular  
regulator modulatory activity.

30 Further provided is a method of identifying the gene and mRNA target sequences targeted by the ribozymes of the invention.

Still further provided is a method of treating an HCV infection by inhibiting the activity of HCV cellular regulators by targeting the regulators with ribozymes or compounds that inhibit the activity of the regulators.

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### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the vector construct and application of a randomized hairpin ribozyme gene vector library for the discovery of HCV IRES regulator molecules.

Figure 2 shows a schematic representation of a hairpin ribozyme gene vector  
10 library.

Figure 3 shows a flow chart of the tissue culture selection system for the discovery of HCV IRES regulator molecules.

Figure 4 shows the selection of 5' tk cells with a GCV-resistant phenotype following transduction with the retroviral hairpin ribozyme gene vector library.

15 Figure 5A shows the nucleotide and amino acid sequence of human eIF2B $\gamma$  (SEQ ID NOS:1 and 2, respectively). Figure 5B shows an amino acid alignment of human and rat eIF2B $\gamma$  (SEQ ID NO:140). Figure 5C shows that Ribozyme RzUCGA 9 (SEQ ID NO:16) targets human eukaryotic initiation factor 2B gamma subunit (eIF2B $\gamma$ ) mRNA.

20 Figure 6 shows quantitation of RNA transcripts in HeLa 5' tk cells by RNA blot analysis.

Figure 7 shows the confirmation of human eIF2B $\gamma$  as a target gene involved in HCV IRES-mediated expression of herpes simplex virus thymidine kinase.

Figure 8 shows a protein blot analysis of HCV Core protein of GCV-resistant  
25 colonies following transduction of single ribozymes into 5' tk cells.

Figure 9 shows the ratio of core protein to RNA transcripts in ribozyme expressing cells.

### DETAILED DESCRIPTION OF THE INVENTION

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The invention is directed to methods of identifying genes required for pathogenesis of infectious diseases and to the identified genes and gene products as therapeutic targets for the treatment of infectious diseases. The methods are directed to

the identification of genes encoding cellular regulators of infectious agents such as Hepatitis C virus (HCV), for example. Genes encoding cellular regulators are sought for identification and as therapeutic targets because they are less susceptible to genetic adaptations, such as mutation to drug resistance, because cellular regulators are also  
5 required in host cell function.

In one embodiment, cellular regulators for HCV have been identified. HCV contains a nucleic acid sequence element present in its RNA that is recognized by cellular translation machinery and is essential for HCV polypeptide expression. The element, termed internal ribosome entry site (IRES), allows for entry of ribosomes and  
10 translation initiation at an internal sequences within an RNA. This translation pathway is distinct from the majority of cellular mRNA which requires the presence of a 5' cap structure for ribosome binding and subsequent translation initiation. A total of eighteen (18) cellular sequences have been identified which correspond to cellular regulators that function in HCV replication or expression. Two of the cellular regulators correspond to  
15 polypeptide subunits of the translation machinery and two of the cellular regulators correspond to a cellular proteasome complex. Inhibiting expression of any of these cellular regulators results in inhibition of IRES-mediated translation of RNA.

As used herein, the term "substantially pure" when used in reference to a nucleic acid or polypeptide of the invention is intended to mean a molecule that is in a form that  
20 is relatively free from cellular components such as lipids, polypeptides, nucleic acids or other cellular material that it is associated with in its natural state.

As used herein, the term "nucleic acid" is intended to mean a single- or double-stranded DNA or RNA molecule. For example, a nucleotide designated as "T" also is equivalent to a "U" nucleotide in a recited sequence. A nucleic acid molecule of the  
25 invention can be of linear, circular or branched configuration, and can represent either the sense or antisense strand, or both, of a native nucleic acid molecule. Unless otherwise indicated, a reference to a nucleotide sequence of a nucleic acid molecule includes the sequence in single stranded form and in double stranded form. A reference to a nucleotide sequence of a nucleic acid molecule also includes reference to its  
30 unrecited complementary strand. The term also is intended to include nucleic acid molecules of both synthetic and natural origin. A nucleic acid molecule of natural origin can be derived from any animal, such as a human, non-human primate, mouse, rat,

rabbit, bovine, porcine, ovine, canine, feline, or amphibian, or from a lower eukaryote, such a *Drosophila*, *C. elegans* or yeast. A synthetic nucleic acid includes, for example, chemical and enzymatic synthesis. The term "nucleic acid" is similarly intended to include analogues of natural nucleotides which have similar binding properties as the referenced nucleic acid and which can be utilized in a manner similar to naturally occurring nucleotides and nucleosides.

As used herein, the term "fragment" when used in reference to a nucleic acid is intended to mean a portion or segment of the nucleic acid molecule having the ability to selectively hybridize or bind to the subject nucleic acid, or its complement. The term "selectively hybridize" as used herein, refers to the ability of a nucleic acid or fragment to bind the subject nucleic acid molecule without substantial crossreactivity with a molecule that is not the subject nucleic acid molecule.

A fragment of a nucleic acid molecule of the invention includes at least about 8-12 nucleotides of the subject nucleic acid. For example, the ribozyme sequence tag (RST) sequences and their corresponding target sequence tags (TST) sequences described herein contain about 8 nucleotides in helix 1 and about 4 nucleotides in helix 2, where about 4 of these nucleotides can lack sequence complementarity to the TST nucleic acid and still exhibit selective hybridization. Therefore, a fragment having the ability to selectively hybridize can contain about 8, 9, 10, 11 or 12 nucleotides of the subject nucleic acid. A fragment can also contain a greater number of nucleotides corresponding to the subject nucleic acid, or complement thereof, including for example, about 13, 14 or 15 nucleotides as well as at least 16, 17, 18, 19 or 20 nucleotides so long as it maintains the ability to selectively hybridize to the subject nucleic acid. Additionally, a fragment can be longer, including at least about 25, 30, 40, 50, 100, 300 or 500 or more nucleotides, and can include up to the full length of the reference nucleic acid molecule minus one nucleotide. Fragments of such lengths are able to selectively hybridize with the subject nucleic acid molecule in a variety of detection formats described herein and known to those skilled in the art. Fragments of the invention expressly exclude known EST sequences available to the public within EST databases as of the date of filing this application.

Therefore, a fragment of a nucleic acid molecule of the invention can be used, for example, as an RST to target a ribozyme to a nucleic acid of the invention; as a PCR primer to selectively amplify a nucleic acid molecule of the invention; as a selective primer for 5' or 3' RACE to determine or identify 5' or 3' sequence of a nucleic acid molecule identified in methods of the invention; as a selective probe to identify or isolate a nucleic acid molecule of the invention on a Northern or Southern blot, or from a genomic or cDNA library; or as a selective inhibitor of HCV replication or expression in a cell infected with HCV. A TST can be used as a PCR primer to selectively amplify a nucleic acid molecule of the invention; as a selective primer for 5' or 3' RACE to determine or identify 5' or 3' sequence of a nucleic acid molecule identified in methods of the invention; as a selective probe to identify or isolate a nucleic acid molecule of the invention on an RNA or DNA blot, or from a genomic or cDNA library.

The term "unique" when used in reference to a specific nucleic acid fragment is intended to mean a fragment of the subject nucleic acid that contains at least one nucleotide at a particular position that is, characteristic, distinct or novel when compared to a different nucleotide sequence, or a related nucleotide sequence at the same or analogous position. In reference to a particular sequence, for example, the human eIF2By nucleotide sequence differs from the rat sequence at about 42 codon positions or about 126 nucleotides within the coding region. Therefore, for each of these codons positions, there is at least one nucleotide which differs from the rat sequence and is therefore characteristic of the human eIF2By nucleotide sequence. An eIF2By nucleic acid fragment containing one such characteristic nucleotide is a unique fragment.

As used herein, the term "substantially the same" when used in reference to a nucleotide sequence is intended to mean that a nucleic acid molecule that retains its ability to selectively hybridize to the reference nucleic acid. Therefore, a nucleic acid molecule having substantially the same sequence compared to a reference nucleic acid can include, for example, one or more additions, deletions or substitutions with respect to the reference sequence so long as it can selectively bind to that sequence. Included within this definition are encoding nucleic acids that have degenerate codon sequences at one or more positions and therefore differ in nucleotide sequence compared to the reference nucleic acid but substantially maintain the referenced encoded amino acid sequence.

As used herein the term "substantially the same" when used in reference to a polypeptide of the invention is intended to mean an amino acid sequence that contains minor modifications with respect to the reference amino acid sequence, so long as the polypeptide retains one or more of the functional activities exhibited by the polypeptide as a whole. A polypeptide that has substantially the same amino acid sequence as a reference human amino acid sequence can be, for example, a homologous polypeptide from a vertebrate species, such as a non-human primate, mouse, rat, rabbit, bovine, porcine, ovine, canine, feline, or amphibian, or from a lower eukaryote, such as *Drosophila*, *C. elegans* or yeast.

A polypeptide that has substantially the same amino acid sequence as a reference sequence can also have one or more deliberately introduced modifications, such as additions, deletions or substitutions of natural or non-natural amino acids, with respect to the reference sequence. Those skilled in the art can determine appropriate modifications that, for example, serve to increase the stability, bioavailability, bioactivity or immunogenicity of the polypeptide, or facilitate its purification, without altering the desired functional activity. For example, introduction of a D-amino acid or an amino acid analog, or deletion of a lysine residue, can stabilize a polypeptide and reduce degradation. Likewise, addition of tag sequences, such as epitope or histidine tags, or sorting sequences, can facilitate purification of the recombinant polypeptide. Depending on the modification and the source of the polypeptide, the modification can be introduced into the polypeptide, or into the encoding nucleic acid sequence.

Computer programs known in the art, for example, DNASTAR software, can be used to determine which amino acid residues can be modified as indicated above without abolishing the desired functional activity. Additionally, guidance in modifying amino acid sequences while retaining functional activity is provided by aligning homologous cellular regulator polypeptides from various species. Those skilled in the art understand that evolutionarily conserved amino acid residues and domains are more likely to play a role in the biological activity than less well-conserved residues and domains.

In general, an amino acid sequence that is substantially the same as a reference amino acid sequence will have greater than about 70% identity, preferably greater than about 85% identity, more preferably greater than about 91% identity, including greater



than about 94% identity with the reference sequence. The amino acid sequences which align across two sequences, and the presence of gaps and non-homologous regions in the alignment, can be determined by those skilled in the art based, for example, on a BLAST 2 or Clustal V or similar computer alignment. A computer alignment, if  
5 desired, can be optimized visually by those skilled in the art. The percent identity of two sequences is determined as the percentage of the total amino acids that align in such an alignment which are identical. Those skilled in the art understand that two amino acid molecules with a given percentage identity over the entire sequence or over a substantial portion or portions thereof, are more likely to exhibit similar functional activities than  
10 two molecules with the same percentage identity over a shorter portion of the sequence. Sequence identity is preferably determined with by BLAST searching with the default settings provided at the website of the National Cancer Biological Institute (NCBI).

As used herein, the term "functional fragment" when used in reference to a polypeptide of the invention is intended to refer to a portion, segment or fragment of the  
15 polypeptide which retains at least one of the activities of the full length polypeptide. For example, a functional fragment of eIF2 $\gamma$  can be a portion of eIF2 $\gamma$  that maintains its ability to bind with one or more other subunits of eIF2 $\gamma$  or a portion of eIF2 $\gamma$  that maintains its ability to facilitate GDP-GTP exchange with eIF2.

As used herein, the term "ribozyme sequence tag" or "RST" is intended to mean  
20 the target recognition domain of a ribozyme. Therefore, the structure of an RST hairpin ribozyme can be 5'-N<sub>8</sub>-AGAA-N<sub>4</sub>-3' where N<sub>8</sub> and N<sub>4</sub> are complementary to sequences of the target RNA. The bases AGAA form a non-binding loop with the NGUC sequence of the target RNA. Therefore, a "target sequence tag" nucleic acid or "TST" as used herein, is a nucleic acid having a nucleotide sequence that is capable of  
25 selectively hybridizing to an RST of a ribozyme and being cleaved by the ribozyme. For example, the TST regions capable of selectively hybridizing to the RST will be substantially the complement of the helix 1 and helix 2 RST region sequences. These selectively hybridizing regions are separated by, for example, a GUC which is capable of being cleaved by a hairpin ribozyme and therefore will have the structure 5'-N<sub>5</sub>-  
30 GUC-N<sub>8</sub>-3' where the first four nucleotides of N<sub>5</sub> represent the TST complementary sequence of the ribozyme helix 2 and N<sub>8</sub> represents the TST complementary sequence of the ribozyme helix 1.

As used herein, the term "ribozyme" or "ribozyme RNA molecule" is intended to mean a catalytic RNA that cleaves RNA. Ribozymes include both hairpin and hammerhead classes which differ in mechanism for hybridization. The term "hairpin ribozyme" is intended to refer to an RNA molecule having the general nucleic acid sequence and two-dimensional configuration of the molecule shown in Figures 1 and 2, and which is capable of selectively hybridizing, or of both selectively hybridizing and cleaving, a target RNA. The term is also intended to include both hairpin ribozyme RNA molecules as well as single- and double-stranded DNA molecules that, when expressed, form hairpin ribozyme RNA molecules. Generally, a hairpin ribozyme will have from about 50 to 54 nucleotides, and forms two helical domains (Helix 3 and Helix 4) and 3 loops (Loops 2, 3 and 4). Two additional helices, Helix 1 and Helix 2, form between the ribozyme and its RNA target or substrate (Figure 2). A hairpin ribozyme binds a target RNA by forming Watson Crick base pairs between the substrate and Helix 1 and Helix 2 sequences, as shown by dots in Figures 1 and 2, where "N" is any nucleotide. The length of Helix 2 is usually about 4 nucleotides, and the length of Helix 1 can vary from about 6-10 nucleotides or more. A hairpin ribozyme can have catalytic activity, and thus cleave the target RNA at the indicated cleavage site in Figure 2. The catalytic activity of the hairpin ribozyme also can be disabled by, for example, altering the AAA sequence in Loop 2 to CGU. Those skilled in the art can determine which modifications to the overall hairpin ribozyme structure can be made and still maintain the target binding, or both target binding and catalytic activity, of a hairpin ribozyme of the invention.

As used herein, the term "library" or "ribozyme gene vector library" is intended to mean a collection or population of different species of ribozyme RNA molecules. Within a population, any of the ribozyme species can be uniquely represented or redundant. Therefore, the term "randomized" or "random" when used in reference to a ribozyme gene vector library is intended to refer to a population of ribozymes that have differing nucleotide sequences in their target recognition sequence. The differing nucleotide sequences can be purposefully introduced, such as by degenerate, partially degenerate or varigated oligonucleotide synthesis, or other methods well known to those skilled in the art. Alternatively, the differing nucleotide sequences can be introduced by a variety of mutagenesis methods, including for example, chemical and enzymatic

methods, known in the art. A random ribozyme gene vector library also can be assembled, for example, from combining a collection of different ribozyme species into a single population. The synthesis and construction of random ribozyme libraries is described further below in the Examples and is the subject matter of U.S. applications 5 60/037,352 and 60/093,828.

As used herein, the term "target recognition sequence" when used in reference to a ribozyme is intended to mean the substrate binding site of a ribozyme, which corresponds to an RST RNA nucleotide sequence or which corresponds to the complement of an TST nucleic acid nucleotide sequence. For the specific example of a 10 hairpin ribozyme, the target recognition sequence corresponds to the nucleotide sequences of the helix 1 or helix 2 domain or both (See Figure 2). The target recognition sequences of helix 1 and 2 can be separated by catalytic nucleotides, which in the specific example of a hairpin ribozyme correspond to the nucleotides AGAA.

As used herein, the term "cellular regulator" when used in reference to virus 15 replication or expression, is intended to mean a gene product, including structural or functional RNA gene products, that are encoded by an endogenous gene of an infected or uninfected cell. Endogenous genes can include, for example, genes originating from the species of the infected cell type as well as heterologous genes that become incorporated into the cell's genome so long as it is not derived from the genome of the 20 infectious agent. To be a cellular regulator, the cellular gene product must also function in the replication or expression of the virus. It is not necessary that the cellular regulator is essential for virus replication or expression, but instead, that the cellular regulator is functionally involved in virus replication or expression.

As used herein, the term "negative selection marker" is intended to mean a gene 25 product that is, or can be made to be cytotoxic or cytostatic to cells. Specific examples include thymidine kinase (tk), cytosine deaminase (CD) and diphtheria toxin (DT). For example, in the presence of nucleoside analogues, the expression of tk and CD is toxic to cells. The cognate compound used in negative selection for the above specific examples include ganciclovir or FIAU for tk and 5-fluorocytosine for cytosine 30 deaminase. Diphtheria toxin is itself toxic because it inhibits protein synthesis and therefore is a noni-conditional marker gene. However, diphtheria toxin can be made

conditional by linking it to an inducible promoter or other regulatory element, for example.

As used herein, the term "treating" when used in reference to an HCV infection is intended to mean a reduction in severity or prevention of an infectious disease.

5 Therefore, "treating an HCV infection" as used herein, is intended to mean a reduction in severity, regression, or prevention of an HCV infection. Reduction in severity includes, for example, an arrest or a decrease in clinical-symptoms, physiological indicators or biochemical markers. Prevention of the infection includes, for example, precluding the occurrence of the infection, such as in prophylactic uses to individuals  
10 susceptible to or suspected of exposure to HCV, or reversing an infected individual to their preinfectious state of health.

The invention provides a substantially pure nucleic acid comprising a nucleotide sequence greater than about 79% identical to SEQ ID NO:1, or a unique fragment thereof. Also provided is a substantially pure polypeptide comprising an amino acid  
15 sequence greater than about 91% identical to SEQ ID NO:2, or functional fragment thereof.

The nucleic acid shown as SEQ ID NO:1 has been found to encode a cellular regulator of HCV which functions in IRES-mediated translation of RNA. As such, it is useful as a target for treating or reducing the severity of HCV or other infectious agents  
20 which utilize IRES elements for replication or expression. Inhibition of the expression or activity of this cellular regulator results in a concomitant decrease in the infecting agent's polypeptide translation and therefore propagation as pathological agent.

SEQ ID NO:1 corresponds to the expressed message of the human gene encoding translation initiation factor 2B gamma subunit (eIF2B $\gamma$ ). SEQ ID NO:1 is  
25 about 1602 nucleotides in length and has 51 and 31 non-coding regions of 102 and 112 nucleotides, respectively. The resultant coding region is 1359 nucleotides in length, coding for a polypeptide of 452 amino acids (Figure 5A). SEQ ID NO:1 has a nucleotide sequence of about 88% identical to the rat sequence within the coding region where no other species of eIF2B $\gamma$  have yet to be identified.

30 Modifications of SEQ ID NO:1 which do not substantially affect the activity of the encoded cellular regulator and which maintain nucleotide sequence identity greater than about 80% are included as nucleic acids of the invention. These nucleic acids

having minor modifications can similarly be used for the development of therapeutic compounds which inhibit the expression or activity of human eIF2 $\gamma$  cellular regulator. Such modifications include, for example, changes in the nucleotide sequence which do not alter the encoded amino acid sequence as well as changes in the nucleotide sequence with result in conservative amino acid substitutions or minor alterations which do not substantially affect the activity of eIF2 $\gamma$ . Those skilled in the art will know or can determine what changes within greater than about 80% compared to SEQ ID NO:1 can be made without substantially affecting the activity of eIF2 $\gamma$  as a cellular regulator of HCV replication or expression.

Unique fragments of SEQ ID NO:1 are also provided. The fragments are useful in a variety of procedures, including for example, as probes for determining the effectiveness of therapeutic agents which target expression of eIF2 $\gamma$  as a cellular regulator of HCV replication or expression, or other IRES-dependent infectious agent. Unique fragments also can be used to encode functional fragments of eIF2 $\gamma$  as therapeutic targets for anti-HCV compounds in the screening methods of the invention. The unique fragments of SEQ ID NO:1 are applicable in a variety of other methods and procedures known to those skilled in the art.

Unique fragments of SEQ ID NO:1 correspond to fragments or portions of SEQ ID NO:1 that are of sufficient length to distinguish the fragment as an eIF2 $\gamma$  encoding nucleic acid and that contain at least one nucleotide characteristic of SEQ ID NO:1. Such a characteristic nucleotide, or nucleotides, within a specific fragment of SEQ ID NO:1 distinguish that fragment from other related nucleotide sequences. For example, fragments of the non-coding region of SEQ ID NO:1 are generally unique when compared to even related nucleotide sequences such as the rat sequence, for example, because there is little evolutionary pressure to conserve non-coding domains. Nucleic acid sequences as small as between about 12-15 nucleotides are statistically unique sequences within the human genome. However, nucleic acids as small as between about 8-12 nucleotides can be unique. Therefore, non-coding region fragments of SEQ ID NO:1 of about 8-9, preferably about 10-11, and more preferably about 12 or 15 nucleotides or more in length can be nucleotide sequences corresponding to unique fragments of SEQ ID NO:1 of the invention.

Additionally, unique nucleotide sequences arise in the coding region of SEQ ID NO:1 as well. Those skilled in the art will know or can determine which nucleotide positions are unique to either a non-coding region or a coding region fragment of SEQ ID NO:1 given the teachings described herein or by alignment (using methods well known to those skilled in the art) of SEQ ID NO:1 with other sequences to be distinguished. Moreover, for ease of reference to distinguishing nucleotides within codon positions, Figure 5B shows an amino acid sequence alignment of the polypeptide encoded by SEQ ID NO:1 with a rat eIF2B $\gamma$  amino acid sequence. The alignment shows that there are about 43 codon differences between these two sequences, indicating at least about 42 or more unique nucleotides in SEQ ID NO:1 compared to the encoding rat nucleotide sequence. Comparison of SEQ ID NO:1 with the nucleotide sequence of rat eIF2B $\gamma$  will reveal that within these 43 codons there are 52 nucleotide differences. Inclusion of any or all of these distinguishing nucleotide differences within a fragment of SEQ ID NO:1 confers uniqueness onto the fragment.

A substantially pure nucleic acid molecule having a nucleotide sequence greater than about 80% identical to SEQ ID NO:1, or a unique fragment thereof, will be of sufficient length and identity to SEQ ID NO:1 to selectively hybridize to it under at least moderately stringent hybridization conditions. For example, it can be determined that a substantially pure nucleic acid molecule contains a nucleotide sequence greater than about 80% as SEQ ID NO:1, or a unique fragment thereof, by determining its ability to hybridize in a filter hybridization assay to a molecule having the sequence of SEQ ID NO:1, but not to other unrelated nucleic acid molecules, under conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C. Suitable alternative buffers and hybridization conditions that provide for moderately stringent hybridization conditions in particular assay formats are known or can be determined by those skilled in the art (see, for example, Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989).

The nucleic acid shown as SEQ ID NO:1 encodes a polypeptide that is 91% identical to rat eIF2B $\gamma$ . The amino acid sequence corresponding to the eIF2B $\gamma$  cellular regulator is shown in SEQ ID NO:2. As with the nucleotide sequence described above, modifications of SEQ ID NO:2 which do not substantially affect the activity of the

cellular regulator and which maintain amino acid sequence identity greater than about 91% are included as polypeptides of the invention. These polypeptides having minor modifications can similarly be used for the development of therapeutic compounds which inhibit the activity of human eIF2By cellular regulator. Such modifications

5 include, for example, changes in the amino acid sequence which do not substantially alter the structure or function of a domain within the polypeptide as well as changes in the amino acid sequence with result in conservative substitutions or minor alterations which do not substantially affect the activity of eIF2By. Those skilled in the art will know or can determine what changes within greater than about 9% compared to SEQ ID

10 NO:2 can be made without substantially affecting the activity of eIF2By as a cellular regulator of HCV replication or expression.

Functional fragments of SEQ ID NO:2 are also provided. The cellular regulator eIF2By is a subunit of a GDP-GTP exchange protein necessary for recharging the cellular regulator eIF2 translation initiation factor with GTP following one cycle of

15 peptide bond formation. Therefore, a specific example of a functional fragment is the domain which binds one or more subunits within the eIF2B complex or the domain which directly or indirectly participates conferring GTP binding specificity onto the eIF2B complex. Other functional fragments of eIF2By also exist and are known, or can be determined by those skilled in the art.

20 The invention also provides a substantially pure TST nucleic acid consisting of a fragment of SEQ ID NO:1 having substantially the nucleotide sequence 5'-N<sub>5</sub>-GUC-N<sub>8</sub>-3' or 5'-N<sub>5</sub>-GUA-N<sub>8</sub>-3' (SEQ ID NOS: 3 and 4, respectively). The TST nucleic acid portion of the fragment can have between about 8-12 nucleotides, and preferably about 9-10 nucleotides at positions N<sub>5</sub> and N<sub>8</sub> that are identical to a fragment of SEQ ID

25 NO:1. Therefore, depending on the length of the TST nucleic acid portion, a fragment of SEQ ID NO:1 as described above can be about 11-15 nucleotides or greater in length.

Hairpin ribozymes cleave RNA substrates 5' to the G nucleotide in either of the sequences 5'-N<sub>5</sub>-GUC-N<sub>8</sub>-3' or 5'-N<sub>5</sub>-GUA-N<sub>8</sub>-3' (SEQ ID NOS: 3 and 4, respectively). Fragments of SEQ ID NO:1 having a corresponding RNA form that is

30 recognized by the target recognition site of a hairpin ribozyme therefore include nucleic acids having the sequence 5'-N<sub>5</sub>-GUC-N<sub>8</sub>-3' or 5'-N<sub>5</sub>-GUA-N<sub>8</sub>-3' (SEQ ID NOS: 3 and 4, respectively) where N<sub>5</sub> and N<sub>8</sub> are nucleotide sequences substantially the same as

a sequence corresponding to SEQ ID NO:1. Such fragments correspond to the complementary sequence of a ribozyme target recognition site or RST and are referred to herein as TST nucleic acids.

The TST nucleic acids can be of any desired length and can include additional sequences other than those corresponding to SEQ ID NO:1 and other moieties so long as they have the structure 5'-N<sub>5</sub>-GUC-N<sub>8</sub>-3' or 5'-N<sub>5</sub>-GUA-N<sub>8</sub>-3' (SEQ ID NOS:3 and 4, respectively) where N<sub>5</sub> and N<sub>8</sub> correspond substantially to a nucleotide sequence of SEQ ID NO:1. Moreover, it is not necessary for all nucleotide residues within the N<sub>5</sub> and N<sub>8</sub> regions to be identical to the corresponding sequence within SEQ ID NO:1. Instead, all that is necessary is for such TST nucleic acids to selectively hybridize to a complementary RST. Therefore, less than all 13 nucleotides at positions N<sub>5</sub> and N<sub>8</sub> can be identical to a nucleotide sequence or fragment of SEQ ID NO:1. Generally, between about 8-12 or between about 9-10 nucleotides are sufficient for selective hybridization of an RST with a TST nucleic acid. Described further below in the Examples is a specific example of an RST present in a ribozyme that selectively hybridizes to SEQ ID NO:1.

Similarly, the TST nucleic acids can be used to design ribozymes that selectively hybridize and cleave an RNA corresponding to SEQ ID NO:1. A specific example of such a ribozyme is a hairpin ribozyme having a target recognition sequence complementary to a TST nucleic acid of SEQ ID NO:1 and having the nucleotide sequence 5'-N<sub>8</sub>-AGAA-N<sub>4</sub>-3' (SEQ ID NO:5). As with the TST nucleic acids described above, it is not necessary that all the sequences with position N<sub>8</sub>, and N<sub>4</sub> be identical in complement to SEQ ID NO:1 so long as the target recognition sequence can selectively hybridize to the RNA form of TST nucleic acid and cleave it as a substrate. Those skilled in the art will know or can determine given the teachings and descriptions herein what RST sequences are sufficient for selective hybridization as well as for cleavage of a target RNA substrate.

Therefore, the invention provides a ribozyme having a target recognition sequence capable of selectively hybridizing to an RNA corresponding to SEQ ID NO:1 and cleaving said RNA. The target recognition sequence of the ribozyme can consist of an RST complementary to a fragment of SEQ ID NO:1 and having substantially the nucleotide sequence 5'-N<sub>8</sub>-AGAA-N<sub>4</sub>-3' (SEQ ID NO:5). The target recognition



sequence can further be between about 8-12 nucleotides, preferably about 9-10 nucleotides at positions N<sub>8</sub> and N<sub>4</sub> that are complementary to a fragment of SEQ ID NO:1.

The invention further provides an RST having one of the following nucleotide sequences:

- 5'–CUAACUUUAGAAACUA–3', 5'–UAAUUAUUAGAAUGCG–3',  
 5'–GCGAUCUAAGAAUCAG–3', 5'–AGACCAAAGAAGCUU–3',  
 5'–ACAGCCAGAGAAACCG–3', 5'–UUAACGCAGAAUACG–3',  
 5'–UAUUGGCUAGAACGAA–3', 5'–UCAGCCUCAGAACUGC–3',  
 10 5'–AGCUGGCAGAACUGC–3', 5'–UUGUUAUAGAAACUU–3',  
 5'–UUCUUAUUAGAAAGCU–3', 5'–UCGCUAAAAGAAGGAA–3',  
 5'–UUCGUCAAAGAAUUCU–3', 5'–UACACGUAGAAAGAC–3',  
 5'–AGCCGAGGAGAAUCCC–3', 5'–CUGUCAACAGAACUCG–3',  
 5'–AUUCAUAUAGAAUGGA–3', 5'–CUUGCGCGAGAACAUC–3',  
 15 5'–AGCCGCAUAGAAGCAG–3', (SEQ ID NOS:6-24, respectively), or a  
 complementary sequence thereof, as described further below. The complementary  
 sequence at positions 9-12 (5'–AGAA–3') can be substituted by the non-  
 complementary ribozyme cleavage sequence 5'–NGUC–3'.

- As described herein, SEQ ID NOS:6-24 represent RST sequences of ribozyme  
 20 binding sites selective for mRNAs whose cleavage resulted in loss of translation of an  
 IRES-dependent RNA. Each of these sequences correspond to a nucleic acid encoding a  
 cellular regulator of HCV replication or expression. As described previously with  
 respect to TST nucleic acid fragments of the cellular regulator shown as SEQ ID NO:1,  
 the 5' terminal N<sub>8</sub> positions and the 3' terminal N<sub>5</sub> positions are separated by the  
 25 intervening trinucleotide sequence 5'–GUC–3' in the cellular regulator RNA and  
 correspond to the complement of the RST sequence or the TST sequence. Also as  
 described previously, at least about 8-12 nucleotides within positions N<sub>8</sub> and N<sub>5</sub> of a  
 TST are sufficient for selective binding between a ribozyme and its cellular regular  
 target RNA. Therefore, a cellular regulator nucleic acid molecule of the invention  
 30 contains at least about 8-12 nucleotides corresponding to an RST sequence set forth as  
 SEQ ID NOS:6-24, or its TST sequence complement corresponding to SEQ ID  
 NOS:25-43, or about 11-15 nucleotides corresponding to an RST sequence set forth as

SEQ ID NOS:6-24, or its TST sequence complement corresponding to SEQ ID NOS:25-43, and including the intervening trinucleotide 5'-GUC-3'.

Therefore, the invention also provides a TST nucleic acid having one of the following nucleotide sequences: 5'-UAGUNGUCAAAGUUAG-3',

- 5 5'-CGCANGUCAAUAAUUA-3', 5'-CUGANGUCUAGAUCGC-3',  
 5'-AAGCNGUCUUUGGUCU-3', 5'-CGGUNGUCCUGGCUGU-3',  
 5'-CGUANGUCGCGUUUAA-3', 5'-UUCGNGUCAGCCAAUA-3',  
 5'-GCAGNGUCGAGGCUGA-3', 5'-GCANGUCUGCCAGCU-3',  
 5'-AAGUNGUCAUUAACAA-3', 5'-AGCUNGUCAUAAGAA-3',  
 10 5'-UUCCNGUCUUAAGCGA-3', 5'-AGAANGUCUUGACGAA-3',  
 5'-GUCUNGUCACGUGUUA-3', 5'-GGGANGUCCCUCGGCU-3',  
 5'-CGAGNGUCGUUGACAG-3', 5'-UCCANGUCAUAUGAAU-3',  
 5'-GAUGNGUCCGCGCAAG-3', 5'-CUGCNGUCAUGCGGCU-3'  
 (SEQ ID NOS:25-43, respectively), or a complementary sequence thereof.

- 15 For simplicity of the description, the cellular regulator nucleic acids of the invention will be described with reference to its TST nucleic acid sequence and specifically with reference to a cellular regulator nucleic acid containing at least about 8-12 nucleotides corresponding to a TST nucleic acid sequence of SEQ ID NOS:25-43. However, it is to be understood that reference to a cellular regulator TST  
 20 nucleotide sequence also specifically includes reference to the complementary sequence of the RST nucleic acid molecule. Therefore, it is also to be understood that reference to a cellular regulator containing at least about 8-12 nucleotides corresponding to a TST sequence of SEQ ID NOS:25-43 also includes reference to a cellular regulator nucleic acid containing at least about 11-15 nucleotides corresponding to a TST sequence set  
 25 forth as SEQ ID NOS:25-43, which includes the intervening trinucleotide 5'-GUC-3' between the 5' terminal N<sub>5</sub> positions and the 3' terminal N<sub>8</sub> positions and as described previously with respect to the cellular regulator shown as SEQ ID NO:1 and its TST fragments. Therefore, the invention also provides substantially pure TST nucleic acids having the structure 5'-N<sub>5</sub>-GUC-N<sub>8</sub>-3' or 5'-N<sub>5</sub>-GUA-N<sub>8</sub>-3' and substantially the  
 30 nucleotide sequences shown as SEQ ID NOS:25-43.

A cellular regulator nucleic acid molecule containing at least about 8-12 nucleotides corresponding to a TST nucleic acid sequence set forth as SEQ ID NOS:25-

43, or about 11-15 nucleotides corresponding to a TST nucleic acid sequence set forth as SEQ ID NOS:25-43, and including the intervening trinucleotide 5'-GUC-3', or a functional fragment thereof, does not have the exact endpoints of nucleotide sequences deposited and available in public databases as of the date of filing the subject application. Such databases include, for example, the non-redundant GenBank database and NCBI dbest EST database. EST sequences for the above-described cellular regulators include GenBank accession numbers: AA703831, AI800599.1, AI632282.1, W58368, W58049, A1660531.1, A1798535.1, AI143649, AI040925, AA102365, AA991764, AA587233, AA580025, A1637675.1, W22190, A1937500.1, T78051, R38705, T90276, T82858, AA507077, Z41323, Z45650, AA649500, T75394, A1700227.1, F01614, A1359536, AA582198, F00408, AA322492, Z24924, F31617.1, AA102364, AA905387, C21034, F05355, AA933569, F34596.1, A1970443.1, AA308943, AA323715, R61886, AA130818, AA311999, AA076401, and AA703831.

A cellular regulator nucleic acid molecule of the invention containing at least about 8-12 nucleotides corresponding to a TST sequence set forth as SEQ ID NOS:25-43, can be advantageously used, for example, as therapeutic targets for the treatment of HCV infections, or to identify and isolate additional sequences corresponding to other regions of the cellular regulator nucleic acid molecules of the invention. When used for the latter purpose, the nucleic acid molecule can contain none, one, or many nucleotides at the 5' or 3' end, or both, of the TST sequences recited as SEQ ID NOS:25-43. These additional nucleotides can correspond to the native sequence of the cellular regulator nucleic acid molecule, or can be non-native sequences, or both. For example, non-native flanking sequences that correspond to a restriction endonuclease site or a tag, or which stabilize the nucleic acid containing at least about 8-12 nucleotides corresponding to a TST nucleic acid sequence set forth as SEQ ID NOS:25-43, in a hybridization assay, can be advantageous when the nucleic acid molecule is used as a probe or primer to identify or isolate longer cellular regulator nucleic acid molecules.

Native cellular regulator nucleotide sequences flanking the at least about 8-12 nucleotides corresponding to a TST sequence set forth as SEQ ID NOS:25-43, can be determined by methods known in the art, such as RT-PCR, 5' or 3' RACE, screening of cDNA or genomic libraries, and the like, using an oligonucleotide having at least about 8-12 nucleotides corresponding to the TST sequence of SEQ ID NOS:25-43 as a primer

or probe, and sequencing the resultant product. The appropriate source of template RNA or DNA for amplification, extension or hybridization screening can be determined by those skilled in the art.

A specific example of a substantially pure cellular regulator nucleic acid molecule containing at least about 8-12 nucleotides of a TST corresponding to SEQ ID NOS:25-43 and flanking coding sequence is the cellular regulator nucleic acid molecule having the nucleotide sequence set forth as SEQ ID NO:1. The isolation of SEQ ID NO:1, based on knowledge of the RST sequence of SEQ ID NO:16, is described further below in the Examples. Moreover, SEQ ID NOS:6, 18 and 24 have similarly been used to identify the nucleic acids for eIF2 $\gamma$  and proteasome alpha subunit as cellular regulators of IRES-dependent translation. Therefore, such procedures can be used to identify and substantially purify longer nucleic acid molecules that contain at least about 8-12 nucleotides corresponding to a TST of SEQ ID NOS:25-43. Such molecules and their functional fragments can be used to produce cellular regulator polypeptides and specific antibodies, for example, by methods known in the art and described herein, for use in the diagnostic and therapeutic methods described herein and known in the art.

As described previously, a cellular regulator nucleic acid molecule, when functionally inactivated in a cell, results in the inhibition of IRES-dependent translation. Such inhibition results in the concomitant decrease in replication or expression of HCV or other IRES-dependent infectious agent. Similar results can be observed by inactivation of the cellular regulator polypeptide by, for example, inhibiting its activity. The cellular regulator activity of a nucleic acid molecule containing at least about 8-12 nucleotides corresponding to a TST of SEQ ID NOS:25-43 and additional native nucleic acid sequences can be further demonstrated using various methods known in the art and described herein. For example, nucleic acid sequences flanking the SEQ ID NOS:25-43 sequences can be selectively targeted in a cell with ribozymes by the methods described herein. The effect on propagation of the infectious agent can be determined by the assays described below. If inactivation by ribozymal cleavage of a second sequence within the isolated nucleic acid molecule also results in a decreased propagation of the infectious agent, that nucleic acid molecule is a confirmed cellular regulator nucleic acid molecule.

Similarly, other types of methods can be used to corroborate the activity of a cellular regulator nucleic acid containing at least about 8-12 nucleotides of a TST corresponding to SEQ ID NO:25-43. For example, an antibody or other selective agent that binds a polypeptide encoded by the nucleic acid molecule can be introduced into the cell, and the effect of the antibody on infection, propagation or IRES-dependent translation of the agent determined. Similarly, an antisense nucleic acid that inhibits transcription or translation of the cellular regulator nucleic acid can be introduced into a cell, and the effect of the antisense nucleic acid on infection or propagation determined. Likewise, an altered form of a cellular regulator nucleic acid molecule, such as a dominant-negative mutant, can be expressed in a cell and its encoded polypeptide will compete with or mimic an endogenous cellular regulator molecule, and thus inhibit infection, propagation or IRES-dependent translation. Those skilled in the art can determine other appropriate assays to demonstrate that a substantially pure nucleic acid molecule containing at least about 8-12 nucleotides of any of SEQ ID NOS:25-43 have cellular regulator activity.

The TST sequences set forth as SEQ ID NOS:25-43 were identified from a random hairpin ribozyme gene vector library by assessing the ability of their corresponding RST to inhibit IRES-dependent translation (SEQ ID NOS:6-24). Therefore, the invention provides ribozymes containing the RST sequences set forth as SEQ ID NOS:6-24 as the ribozyme target recognition sequence. The hairpin ribozymes, of the invention selectively bind to cellular regulator mRNA molecules complementary, in part, to these RST sequences.

A substantially pure hairpin ribozyme of the invention can be catalytic, so as to bind and cleave a cellular regulator nucleic acid messenger RNA. A catalytic hairpin ribozyme of the invention can therefore be used to selectively regulate the activity of a cellular regulator nucleic-acid molecule of the invention. A substantially pure hairpin ribozyme of the invention can also be catalytically disabled, for example, by replacement of the Loop 2 AAA sequence indicated in Figure 2 with a UGC sequence, so as to bind, but not cleave, a cellular regulator nucleic acid molecule of the invention. A non-catalytic hairpin ribozyme can be used, for example, as a control for the inhibition activity of non-disabled ribozymes.

Therefore, the invention also provides a ribozyme containing a target recognition sequence having any one of the following nucleotide sequences:

- 5'–CUAACUUUAGAAACUA–3', 5'–UAAUUAUUAGAAUGCG–3',  
5'–GCGAUCUAAGAAUCAG–3', 5'–AGACCAAAGAAGCUU–3',  
5'–ACAGCCAGAGAAACCG–3', 5'–UUAACGCAGAAUACG–3',  
5'–UAUUGGCUAGAACGAA–3', 5'–UCAGCCUCAGAACUGC–3',  
5'–AGCUGGCAGAACUGC–3', 5'–UUGUUAUAGAAACUU–3',  
5'–UUCUUAUUAGAAAGCU–3', 5'–UCGCUUAAAGAAGGAA–3',  
5'–UUCGUCAAAGAAUUCU–3', 5'–UAACACGUAGAAAGAC–3',  
5'–AGCCGAGGAGAAUCCC–3', 5'–CUGUCAACAGAACUCG–3',  
5'–AUUCAUAUAGAAUGGA–3', 5'–CUUGCGCGAGAACAUC–3',  
5'–AGCCGCAUAGAAGCAG–3' (SEQ ID NOS:6-24, respectively).

- The nucleic acid molecules of the invention, including cellular regulator nucleic acid molecules and fragments, and hairpin ribozyme nucleic acid molecules, can be produced or isolated by methods known in the art. The method chosen will depend, for example, on the type of nucleic acid molecule one intends to isolate. Those skilled in the art, based on knowledge of the nucleotide sequences described herein, can readily isolate cellular regulator nucleic acid molecules as genomic DNA, or desired introns, exons or regulatory sequences therefrom; as full-length cDNA or desired fragments therefrom; or as full-length mRNA or desired fragments therefrom, by methods known in the art. Likewise, those skilled in the art can produce or isolate hairpin ribozymes selective for these sequences.

- A useful method of isolating a cellular regulator nucleic acid molecule of the invention involves amplification of the nucleic acid molecule using the polymerase chain reaction (PCR), and purification of the resulting product by gel electrophoresis. For example, either PCR or reverse-transcription PCR (RT-PCR) can be used to produce a cellular regulator nucleic acid molecule having any desired nucleotide boundaries. Desired modifications to the nucleic acid sequence can also be introduced by choosing an appropriate primer with one or more additions, deletions or substitutions. Such nucleic acid molecules can be amplified exponentially starting from as little as a single gene or mRNA copy, from any cell, tissue or species of interest. An

example of the isolation of a cellular regulator nucleic acid molecule using PCR is presented below in the Examples.

A further method of producing or isolating a cellular regulator nucleic acid molecule of the invention is by screening a library, such as a genomic library, cDNA  
5 library or expression library, with a detectable agent. Such libraries are commercially available or can be produced from any desired tissue, cell, or species of interest using methods known in the art. For example, a cDNA or genomic library can be screened by hybridization with a detectably labeled nucleic acid molecule having a nucleotide  
10 sequence disclosed herein. Additionally, an expression library can be screened with an antibody raised against a polypeptide corresponding to the coding sequence of a cellular regulator nucleic acid disclosed herein. The library clones containing cellular regulator nucleic acid molecules of the invention can be purified away from other clones by methods known in the art.

Furthermore, nucleic acid molecules of the invention can be produced by  
15 synthetic means. For example, a single strand of a nucleic acid molecule can be chemically synthesized in one piece, or in several pieces, by automated synthesis methods known in the art. The complementary strand can likewise be synthesized in one or more pieces, and a double-stranded molecule made by annealing the complementary strands. Direct synthesis is particularly advantageous for producing  
20 relatively short molecules, such as RST or hairpin ribozyme nucleic acid molecules, as well as hybridization probes and primers.

If it is desired to subclone, amplify or express a substantially pure nucleic acid molecule of the invention, the isolated nucleic acid molecule can be inserted into a commercially available cloning or expression vector using methods known in the art.  
25 Appropriate regulatory elements can be chosen, if desired, to provide for constitutive, inducible or cell type-specific expression in a host cell of choice, such as a bacterial, yeast, amphibian, insect or mammalian cell, including human cells. Those skilled in the art can determine an appropriate host and vector system for cloning a nucleic acid molecule of the invention or for expressing and purifying its encoded polypeptide.

30 Methods for introducing a cloning or expression vector into a host cell are well known in the art and include, for example, various methods of transfection such as the calcium phosphate, DEAE-dextran and lipofection methods, viral transduction,

electroporation and microinjection. Host cells expressing cellular regulator nucleic acid molecules can be used, for example, as a source to isolate recombinantly expressed cellular regulator polypeptides, to identify and isolate molecules that regulate or interact with cellular regulator nucleic acids and polypeptides, or to screen for compounds that enhance or inhibit the activity of a cellular regulator molecule of the invention, as described further below.

The methods of isolating, cloning and expressing nucleic acid molecules of the invention described herein are routine in the art and are described in detail, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York(1992) and in Ansubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1989) .

Cellular regulator polypeptides and functional fragments of the invention can be isolated or prepared by methods known in the art, including biochemical, recombinant and synthetic methods. For example, a cellular regulator polypeptide can be purified by routine biochemical methods from a cell or tissue source that expresses abundant amounts of the corresponding transcript or polypeptide. Biochemical purification can include, for example, steps such as solubilization of the appropriate tissue or cells, isolation of desired subcellular fractions, size or affinity chromatography, electrophoresis, and immunoaffinity procedures. The methods and conditions for biochemical purification of a polypeptide of the invention can be chosen by those skilled in the art, and purification monitored, for example, by an ELISA assay or a functional assay.

A fragment having any desired boundaries and modifications to a cellular regulator amino acid sequences can also be produced by recombinant methods. Recombinant methods involve expressing a nucleic acid molecule encoding the desired polypeptide or fragment in a host cell or cell extract, and isolating the recombinant polypeptide or fragment, such as by routine biochemical purification methods described above. To facilitate identification and purification of the recombinant polypeptide, it can be desirable to insert or add, in-frame with the coding sequence, nucleic acid sequences that encode epitope tags, polyhistidine tags, glutathione-S-transferase (GST) domains, and similar sequences that direct expression of the polypeptide in the periplasm or direct



secretion. Methods for producing and expressing recombinant polypeptides *in vitro* and in prokaryotic and eukaryotic host cells are well known in the art..

Functional fragments of a cellular regulator polypeptide can also be produced, for example, by enzymatic or chemical cleavage of the full-length polypeptide. Methods  
5 for enzymatic and chemical cleavage and for purification of the resultant peptide fragments are well known in the art (see, for example, Deutscher, Methods in Enzymology, Vol. 182, "Guide to Protein Purification," San Diego: Academic Press, Inc. (1990)).

Furthermore, functional fragments of a cellular regulator polypeptide can be  
10 produced by chemical synthesis. If desired, such as to optimize their functional activity, stability or bioavailability, such molecules can be modified to include D-stereoisomers, non-naturally occurring amino acids, and amino acid analogs and mimetics. Examples of modified amino acids and their uses are presented in Sawyer, Peptide Based Drug Design, ACS, Washington (1995) and Gross and Meienhofer, The Peptides: Analysis,  
15 Synthesis, Biology, Academic Press, Inc., New York (1983).

A functional activity of a cellular regulator polypeptide or fragment of the invention can be its ability to alter, such as inhibit, IRES-dependent translation when expressed or introduced in a cell. To determine whether a given polypeptide or fragment has the ability to alter IRES-dependent translation, a polypeptide or fragment can be  
20 expressed in the cell by recombinant methods known in the art and the effect of the cellular regulator can be determined *in vitro*. Alternatively, expression of the cellular regulator can be inhibited *in vivo*, including cell culture or animal models and the replication or expression of the infectious agent can be determined. Similarly, expression of the cellular regulator can be inhibited *in vivo*, including cell culture or  
25 animal models and the expression of an IRES-dependent reporter marker determined. A decrease in the replication or expression of the infectious agent or in the expression of an IRES-dependent reporter marker indicates that the polypeptide or fragment is a cellular regulator of the invention.

The invention further provides a method of identifying a compound that inhibits  
30 the activity of a cellular regulator. The method consists of contacting a sample containing a cellular regulator and a nucleic acid element acted on by the cellular regulator with a test compound under conditions that allow replication or expression of

the nucleic acid element or a gene or mRNA operatively linked to the nucleic acid element, and measuring the amount of replication or expression of the nucleic acid element, the gene or mRNA, a decrease in the amount of replication or expression in the presence of the test compound compared to the absence of the test compound indicating  
5 that the compound has cellular regulator inhibitory activity. The magnitude of the decrease in replication or expression activity will correlate with the cellular regulator inhibitory activity of the test compound.

Similarly, compounds that increase or enhance the activity of cellular regulator also can be identified. A test compound added to a sample containing a cellular  
10 regulator and a nucleic acid element acted on by the cellular regulator which increases the amount or rate of replication or expression of the nucleic acid or a gene operatively linked to the nucleic acid element compared to the absence of the test compound indicates that the compound increases the activity of the cellular regulator. Therefore, the invention provides a method of identifying compounds that modulate the activity of  
15 a cellular regulator.

A reaction system for identifying a compound that inhibits or increases cellular regulator activity can be prepared using essentially any sample, material or components thereof that contains a cellular regulator. A cellular regulator containing sample used for such methods can be, for example, *in vitro* transcription or translation systems using, for  
20 example, nucleic acid derived from the infectious agent or a hybrid construct linking the nucleic acid element acted on by a cellular regulator to a reporter gene. Alternatively, cellular nucleic acids and proteins can also be used since the cellular regulator also acts on nucleic acid elements of the host machinery. The cellular regulator containing sample can additionally be derived from cell extracts, cell fractions or, for example, *in*  
25 *vivo* systems such as cell culture or animal models which contain a nucleic acid element acted on by a cellular regulator. The replication levels of these nucleic acids, or the expression levels or activity of encoded products derived from the infectious agent or the reporter gene can be measured in the reaction system to determine the modulatory effect of the test compound on the cellular regulator. Such measurements can be  
30 determined using methods described herein as well as methods well known to those skilled in the art.

Briefly, the cellular regulator source is combined with a nucleic acid element or protein acted on by the cellular regulator as described above and incubated in the presence or absence of a test compound. The amount or rate of replication or expression from the nucleic acid in the presence of the test compound is compared with that in the absence of the test compound. Those test compounds which provide inhibition of replication or expression of at least about 50% are considered to be cellular regulator inhibitors, or antagonists, and further as potential therapeutic compounds for the treatment infectious diseases mediated by the donor agent of the nucleic acid element.

Similarly, those compounds which increase the replication or expression by about two-fold or more are considered to be compounds which increase the activity of a cellular regulator (a cellular regulator agonist). Such agonists can be used as therapeutics, for example, to shift the balance of cellular machinery use away from the replication or expression of the infectious agent. Compounds identified to modulate cellular regulator activity can, if desired, be subjected to further *in vitro* or *in vivo* studies to corroborate that they affect the activity of a cellular regulator toward the replication or expression of an infectious agent.

Suitable test compounds for the above-described assays can be any substance, molecule, compound, mixture of molecules or compounds, or any other composition which is suspected of being capable of inhibiting or enhancing cellular regulator activity *in vivo* or *in vitro*. The test compounds can be macromolecules, such as biological polymers, including proteins, polysaccharides and nucleic acids. Sources of test compounds which can be screened for cellular regulator inhibitory activity include, for example, libraries of small organic molecules, peptides, polypeptides, DNA, and RNA. Additionally, test compounds can be pre-selected based on a variety of criteria. For example, suitable test compounds can be selected as having known inhibition or enhancement activity with respect to translation or proteasome function, for example. Alternatively, the test compounds can be selected randomly and tested by the screening methods of the present invention. Test compounds can be administered to the reaction system at a single concentration or, alternatively, at a range of concentrations to determine, for example, the optimal modulatory activity toward the cellular regulator.

The invention provides a method of identifying a ribozyme reactive with a cellular regulator of virus replication or expression. The method consists of: (a)

introducing a randomized ribozyme gene vector library into a population of cells expressing a negative selection marker gene operatively linked to a viral nucleic acid element acted on by a cellular regulator of virus replication or expression; (b) subjecting said population of cells to negative selection, and (c) recovering one or more ribozymes from viable cells following said negative selection.

Also provided is a method of identifying a cellular regulator of virus replication or expression. The method consists of: (a) introducing a randomized ribozyme gene vector library into a population of cells expressing a negative selection marker operatively linked to a viral nucleic acid element acted on by a cellular regulator of virus replication or expression; (b) subjecting said population of cells to negative selection; (c) recovering one or more ribozymes from viable cells following said negative selection; and (d) sequencing the target recognition sequence of said recovered ribozyme to identify the nucleic acid encoding said cellular regulator.

By reference to a virus, or to HCV in particular, as an exemplary infectious agent amenable to the methods of identifying a ribozyme or a cellular regulator of the invention, one skilled in the art will readily know, in light of the teachings and description herein that such methods are applicable to essentially all infectious agents which require cellular regulators for continued life cycle propagation including, for example, Hepatitis A, Hepatitis G, rhinovirus and poliovirus. Therefore, the methods of identifying a cellular regulator, or ribozyme selective to a cellular regulator, as well as methods of treating an infectious disease once such regulators have been identified are applicable to a variety of infectious diseases including, for example, both DNA and RNA viral infections and parasitic diseases.

For the successful application of such methods, it is sufficient to have identified a nucleic acid derived from an infectious agent which is acted on by a cellular regulator. Once identified, the nucleic acid derived from the infectious agent can be operatively linked to a negative selection marker gene, for example, and subjected to negative selection using the methods of the invention.

Moreover, it is not necessary for the nucleic acid derived from the infectious agent to be unique to the replication or expression mechanism of that agent. Instead, the infectious agent derived nucleic acid acted on by a cellular regulator can include components or structures of cellular replication or expression element and therefore

overlap or be redundant with cellular machinery. The replication or expression efficiency of an infectious agent can rely on a balance between its own and cellular machinery for successful propagation. Therefore, decreasing the level or activity of cellular regulators acting on common components or structures can shift the balance toward utilization of cellular regulators for cellular functions at the expense of the infectious agent. An example of a nucleic acid acted on by a cellular regulator that includes a common component with cellular elements is a viral transcription promoter. Both the viral and cellular element are acted on by the RNA polymerase II cellular protein complex. A specific example of a nucleic acid acted on by a cellular regulator that is distinctive to an infectious agent is a viral IRES sequence because few cellular genes are expressed using this type of nucleic acid element.

The method of identifying a ribozyme reactive with a cellular regulator of virus replication, or its corresponding cellular regulator, involves the construction of a population of cells expressing a negative selection marker gene which is under the control, or operatively linked to a nucleic acid acted on by a cellular regulator of virus replication or expression. A specific example of such a cell population and its use is described further below in the Examples.

Briefly, the nucleic acid element acted on by a cellular regulator can be essentially any viral sequence found in cis on the viral nucleic acid but acted on in trans by a cellular regulator. A specific example, of such an element is the HCV IRES element. This element is also found in other flaviviruses as well as in rhinoviruses, encephalomyocarditis virus, foot-and-mouth disease virus, coxsackievirus and infectious bronchitis virus, for example, and as such, methods using cellular regulators identified for the HCV IRES element and therapeutic compounds thereto are applicable to all of the above-recited viruses and their corresponding diseases. Other elements can include, for example, viral promoters, enhancers and viral replication elements. Those skilled in the art will know what cis acting viral elements are applicable in the methods of the invention.

Cell populations containing cis acting nucleic acid element acted on by a cellular regulator are operatively linked to a negative selection marker gene. Operative linkage will depend on the type of element employed and is intended to refer to placing the viral element in an appropriate context and location in the reporter construct as it would be

found in its native genome. In the specific example of an IRES element, operative linkage places the element 3' to the transcription start site and 5' to the start codon. Similarly, operative linkage of a promoter element will be sufficiently upstream of the translation start codon to include sufficient 5' untranslated region sequence to effect translation in, for example, a CAP-dependent manner. The reporter constructs can be introduced into cell population using well known methods in the art and as described previously.

A negative selection marker can be a gene product that is, or can be made to be cytotoxic or cytostatic to cells. Specific examples include thymidine kinase (tk), cytosine deaminase (CD) and diphtheria toxin (DT). For example, the expression of these selection markers in cells is either toxic alone, or toxic in the presence of a selection compound which is metabolized by the marker gene product into a cytotoxic or cytostatic substance.

For example, ganciclovir is a purine nucleoside analogue having the structure [9- (1, 3-dihydroxy-2 -propoxy) methyl] guanine. FIAU is a pyrimidine nucleoside analogue having the structure 1-(2"-deoxy-2'-fluoro-1- $\beta$ -D-arabinofuranosyl-5-ido) uracil. These compounds are phosphorylated by the tk gene product, leading to their incorporation into replicated DNA during S phase, and subsequent cell death. Ganciclovir and FIAU are about 1000-fold better substrates for the Herpes simplex virus thymidine kinase (HSV-tk) compared to mammalian tk. Concentrations used for selection are between about 0.5-10  $\mu$ M, and preferably about 2  $\mu$ M for ganciclovir whereas FIAU concentrations are between about 0.05-1.0  $\mu$ M, and preferably about 0.2  $\mu$ M.

Negative selection with cytosine deaminase can employ, for example, the compound 5-fluorocytosine. This purine analogue is converted to 5-fluorouracil in the presence of cytosine deaminase which is incorporated into DNA, resulting in cell death. Concentrations used for 5-fluorocytosine are between about 50-250  $\mu$ g/ml.

Finally, diphtheria toxin is itself toxic because it inhibits protein synthesis through NAD-dependent ADP-ribosylation of elongation factor 2 and, therefore, is a non-conditional marker gene. However, linkage of a diphtheria toxin gene to an inducible promoter or other regulatory element can make its expression conditional and therefore amenable to the methods of the invention.

Once negative selection proceeds, the surviving cells are those which express a ribozyme that is reactive with a cellular regulator required for replication or expression of the virus. The cells are isolated and the ribozymes are recovered using, for example, PCR or other well known methods in the art. The RST of the ribozyme is a sequence tag  
5 corresponding to a cellular regulator for virus replication or expression. Sequencing of this tag identifies the nucleic acid encoding the cellular regulator. Specific examples of RSTs corresponding to cellular regulators of the invention are set forth in Figure 4D and as SEQ ID NOS:6-24, 72 and 73, and their corresponding TSTs are set forth as SEQ ID NOS:25-43. The function of four of these sequences have been determined.

10 Specifically, SEQ ID NO:16 corresponds to the RST for human eIF2B $\gamma$ , the full length nucleotide and amino acid sequences of which are shown as SEQ ID NOS:1 and 2. SEQ ID NO:6 corresponds to the RST for human eIF2 $\gamma$  while SEQ ID NOS:18 and 24 correspond to the RSTs for human proteasome alpha subunits PSMA1 and PSMA7, respectively

15 The invention also provides a method of treating an HCV infection. The method consists of introducing a ribozyme selectively reactive with an RNA encoding a subunit of a eukaryotic translation initiation factor or a proteasome into a cell infected with HCV. Also provided is a method of treating HCV by introducing a ribozyme selectively reactive with an RNA encoding a cellular regulator corresponding to an RST selected  
20 from the group consisting of SEQ ID NOS:6-24.

By substituting the ribozymes of the invention selectively reactive with a cellular regulator RNA with an antisense nucleic acid corresponding to an RST sequence selected from the group consisting of SEQ ID NOS:6-24, methods of treating an HCV infection are also provided. The antisense nucleic acids hybridize, for  
25 example, to the cellular regulator nucleic acid similar to catalytic ribozymes, and inhibit transcription processing or translation of the RNA without subsequent cleavage. Such methods will be described below with reference to a ribozyme of the invention, but those skilled in the art will know that antisense nucleic acids can similarly be substituted for the ribozymes to prevent or reduce the severity of an HCV infection.

30 A ribozyme encoding any of the RST sequences set forth as SEQ ID NOS:6-24, or a combination thereof can be delivered in a wide variety of ways to HCV-infected or HCV-susceptible cells to interrupt or prevent HCV infection. The ribozyme can be

administered as RNA or expressed from an expression vector. The ribozyme can be administered *ex vivo* by, for example, administering to cells that have been removed from an infected individual, and then returned to the individual, or the ribozyme can be administered *in vivo*. Delivery can be performed using any appropriate delivery vehicle  
5 known to those skilled in the art including, for example, a liposome, a controlled release vehicle, electroporation or covalently attached moieties, and other pharmacologically acceptable methods of delivery. A carrier can provide specificity for liver accumulation of the ribozyme at the liver which is the primary site of HCV infection. The ribozyme delivery vehicle can be designed to serve as a slow release reservoir or to deliver its  
10 contents directly to the target cell. Examples of ribozyme delivery vehicles include liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. Liposomes can readily be targeted to the liver for delivery of RNA to infected hepatocytes.

Routes of ribozyme administration include intramuscular, aerosol, intravenous,  
15 parenteral, intraperitoneal. Generally however, the route of administration will be through the portal vein since this is a direct route to the liver. The dosage of ribozyme will also depend on a variety of factors, such as the form of the ribozyme, the route of administration, the severity of infection or stage of disease, the general condition of the patient being treated, and thus can vary widely. Generally the dosage of ribozyme will  
20 be between about 10 µg - 200 mg/kg of body weight per day and result in therapeutic or prophylactic levels within the targeted cells sufficient to inhibit or eradicate HCV from the cells. The duration of treatment may extend throughout the course of HCV infection or disease symptoms, usually at least about 7-30 days, with a longer duration being necessary for severe infections. The number and timing of doses can also vary  
25 depending on, for example, the extent of infection.

A viral vector containing a ribozyme corresponding to a cellular regulator RST of the invention can be prepared in any of a wide variety of ways known to those skilled in the art. Representative retroviral vectors which can be used in the methods of the invention are described, for example, in U.S. Patent Nos. 4,861,719, 5,124,263 and  
30 5,219,740. Other vectors may also be employed, particularly for the *ex vivo* methods, such as DNA vectors, pseudotype retroviral vectors, adenovirus, and adeno-associated virus vectors.



The viral vector, consisting of infectious, but replication-defective, viral particles, which contain at least one DNA sequence encoding a ribozyme selectively reactive with a cellular regulator, is administered in an amount effective to inhibit or prevent HCV infection in a host. The vector particles may be administered in an amount  
5 from 1 plaque forming unit to about  $10^{14}$  plaque forming units, more preferably from about  $1 \times 10^6$  plaque forming units to about  $1 \times 10^{13}$  plaque forming units. A sufficient number of vector particles containing a ribozyme selectively reactive with a cellular regulator of the invention is administered to the liver to infect up to at least about 50% of the hepatocytes, usually about 80%, preferably about 90%, or more of the  
10 hepatocytes in the individual. Subsequent administrations can be performed, as needed, to effectively treat or reduce the severity of the HCV infection.

Exemplary ribozymes of the invention include, for example, those having RST sequences set forth as SEQ ID NOS:6-24. Two of these RST sequences have sequence complementary to two different translation initiation factor subunits, namely, eIF2B $\gamma$   
15 and eIF2 $\gamma$ . Another two of these RST sequences have sequence complementary to different subunits of a proteasome complex, namely, proteasome alpha subunit PSMA1 and PSMA7. The RST sequences corresponding to these specific cellular regulators are set forth as SEQ ID NOS:6, 16, 18 and 24, respectively.

In addition to the methods of treating an HCV infection using ribozymes of the  
20 invention, inhibitory compounds identified by the screening methods described previously can be used to reduce the severity of such an infection. Small organic compounds have particular advantage because of their ease of formulation and administration using well known methods in the pharmaceutical arts.

It is understood that modifications which do not substantially affect the activity  
25 of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

## Preparation of the random retroviral vector ribozyme gene vector library

5           This example demonstrates the construction of a random retroviral plasmid ribozyme gene library. The inventors have discovered that by introducing a random retroviral plasmid ribozyme gene library into a HeLa cell line having a HSV thymidine kinase gene linked to an internal ribosome entry site (IRES), certain of the ribozymes will selectively target and inactivate mRNA molecules necessary for IRES-dependent  
10 translation. If the ribozyme has inactivated IRES-dependent translation, the HeLa cells will survive ganciclovir-mediated killing. The ribozyme genes are then rescued from the surviving colonies and sequenced across their substrate binding sites. The corresponding ribozyme binding sequence, or "target sequence tag" (TST) is a sequence present in an IRES-dependent translation regulator nucleic acid molecule targeted by  
15 the ribozyme. Thus, knowledge of the TST allows novel IRES-dependent translation regulator nucleic acids to be identified and isolated.

A plasmid-based retroviral library was constructed by inserting random ribozyme gene sequences into parent vector pLHPM-2kb. pLHPM-2kb contains 5' and 3' long terminal repeats (LTR) of the Moloney retroviral genome; a neomycin resistance  
20 gene driven by the LTR; an SV40 promoter driving a puromycin resistance gene; and a ribozyme transcription cassette containing a tRNA<sup>val</sup> promoter and a 2 kb stuffer insert. When the stuffer insert is removed and replaced by the random ribozyme gene vector library inserts, the tRNA<sup>val</sup> promoter can drive transcription of the inserted ribozyme gene.

25           To prepare the pLHPM-2kb vector, plasmid pLHPM was digested overnight at 65°C with BstB1, phenol: chloroform extracted and ethanol precipitated. The resuspended DNA was then digested overnight at 37°C with MluI. This double digestion excises the 2kb stuffer fragment. The resultant 6kb plasmid vector DNA fragment was purified by agarose gel electrophoresis.

30           To prepare the random ribozyme gene vector library inserts, three oligonucleotides were synthesized and annealed in annealing buffer (50 mM NaCl, 10 mM Tris pH 7.5, 5 mM MgCl<sub>2</sub>) at a molar ratio of 1:3:3 (oligo1:oligo2:oligo3) by

heating to 90°C followed by slow cooling to room temperature. The three oligonucleotides had the following sequences:

Oligol: 5'-cgcgtaggtaataaccacacgtgtgttctctggtnnn

5 nttctnnnnnnnnnggatcctgtttccgcccgggtt-31 (SEQ ID NO:44)

Oligo2: 5'-cggtgtggtatattacctggta-3' (SEQ ID NO:45)

Oligo3: 5'-cgaaaccggcggaacagg-3' (SEQ ID NO:46)

10

To provide for random and uniform incorporation of A, T, C and G nucleotides at the positions represented as N nucleotides in oligol, the A, T, C and G reagents were premixed, and the same mixture used for every N position in the oligonucleotide synthesis. The ribozyme insert library formed by annealing the three oligonucleotides (SEQ ID NOS:44-46) thus contains 8 positions with random nucleotides corresponding to helix 1 of the ribozyme, and 4 random positions with random nucleotides corresponding to helix 2 of the ribozyme.

In order to ligate the pLHPM-RzLib vector DNA fragment with the random ribozyme insert library, 0.5 pmole of the vector and an 8-fold molar excess of annealed oligonucleotides were ligated overnight with 10 units of T4 DNA ligase. Ultracompetent DH12S bacteria (Life Technologies) were then electroporated with the ligation mixture. Bacterial colonies containing the retroviral plasmid ribozyme gene vector library were obtained.

The bacterial colonies containing the retroviral plasmid ribozyme gene vector library were pooled in aliquots as a master stock and frozen at -80°C. Working stocks were made by culturing 1 ml of the master stock in 60 ml LB media overnight at 30°C. A 1 ml aliquot of the working stock was used to make a 500 ml bacterial culture by incubation at 30°C overnight. Plasmid DNA was then-extracted from the 500 ml culture and transfected into HF revertant cells, as described in Example II, below.

30 Following the cloning of the randomized hairpin ribozyme genes into pLHPM, the "randomness" of the plasmid library was evaluated by both statistical and functional analyses. A complete ribozyme gene vector library of this design, with 12 random

positions, would contain  $4^{12}$ , or  $1.67 \times 10^7$ , different members. For the statistical analysis, forty individual bacterial transformants were picked and sequenced. This allowed an evaluation of the complexity of the library, without having to manually sequence each library member. The statistical "randomness" of the library was  
5 determined utilizing the formula for a two-sided approximate binomial confidence interval:  $E = 1.96 \times \sqrt{P(1-P)/N}$ , where  $P$  = the expected proportion of each nucleotide in a given position (this value for DNA bases equals 25% or  $P=0.25$ );  $E$  = the desired confidence interval around  $P$  (i.e.  $P \pm E$ ); and  $N$  = the required sample size (Callahan Associates, Inc., La Jolla, CA). To determine the proportion of each base  
10 within 5% ( $E=0.05$ ), the required sample size is 289. Since each ribozyme molecule contains twelve independent positions, the number of individual ribozyme genes that need to be sequenced out of the library equals 289 divided by 12, or about 25 molecules.

For a functional evaluation of the library's complexity, *in vitro* cleavage was  
15 utilized to determine if ribozymes that target known RNA substrates were present in the library pool. This involved *in vitro* transcribing a comparable ribozyme gene vector library in one reaction and then testing the pool's ability to cleave a variety of different RNA substrates. Several different short RNA targets were properly and efficiently cleaved by the *in vitro* transcribed library. This qualitative analysis suggested a  
20 significantly complex library of ribozyme genes.

Viral vector was produced from the ribozyme gene vector library plasmid using a triple transfection technique. CF2 cells were seeded at  $3.5 \times 10^4$  cells/cm<sup>2</sup> one day prior to transfection. The cells were transfected with a 1:1:1 mixture of the ribozyme gene vector library plasmid or control ribozyme plasmid, a plasmid encoding the  
25 moloney-murine virus gag-pol genes, and a plasmid encoding the vesicular stomatitis virus-G gene, using the cationic lipid TransIT-LT1 (Pan Vera Corporation).  $7.8 \times 10^6$  cells were transfected with 25 mg of each plasmid complexed with 250 ml of the lipid in a total volume of 20 ml of serum free media. After 6 hours, the media was replaced with growth media. The cell supernatant containing retroviral particles was collected  
30 every 24 hours beginning on day 2 after addition of fresh media. The supernatant was filtered through 0.4  $\mu$ m filters and titered in a standard assay using HT1080 cells.

## EXAMPLE II

### Isolation of ribozymes that target IRES-dependent translation factors

This example demonstrates the isolation of ribozyme genes that confer  
5 ganciclovir (GCV) resistance by inactivating IRES-dependent translation, and the  
identification of the nucleic acid sequences they target.

The HeLa cell line used in these experiments was modified with a bicistronic  
reporter gene that confers hygromycin B resistance and GCV resistance. The bicistronic  
reporter gene pHyg-5' tk was constructed with the SV40 promoter driving the  
10 expression of hygromycin B phosphotransferase and the 5' untranslated region of HCV  
(nucleotides (nt) 38-341) upstream of HSV thymidine kinase (HSV-tk, see Figure 1).  
The 5' nontranslated region of hepatitis C virus (HCV) functions as an internal ribosome  
entry site (IRES) and is essential for translation of HCV proteins. Transcription of the 5'  
nontranslated region allows for IRES-mediated translation of HSV-tk, which converts  
15 GCV to the monophosphate which is further metabolized to the triphosphate, a  
cytotoxic analog of GTP. Cells that express ribozymes whose target mRNAs encode  
proteins necessary for IRES-dependent translation will no longer synthesize HSV-tk  
and therefore will survive GCV selection.

In the synthesis of pHyg-5' tk, the hygromycin B phosphotransferase gene was  
20 amplified from pIRES hygro (Clontech) by PCR with oligonucleotide primers P1 (5'-  
ggatgat-gaagacat-acaaggagacgacctccatggatagatccggaaagcct-3'; SEQ ID NO:66) and P2  
(5'-gtcggcatgtcgactattccttggccctcgagc-3'; SEQ ID NO:67), then digested with BbsI  
and SalI and used to replace the puromycin-resistance gene in pPur-HCV (Welch et  
al., 1996) after digestion with BsmBI and SalI to generate pHyg-5'C. The herpes simplex  
25 virus thymidine kinase gene was amplified by PCR with primers P3 (5'-  
cgatcgtagaattcaggtctcgtagaccgtgcacatggcttcgtaccctgccatcaacacgcgtctgcgttcgaccaggct-  
3'; SEQ ID NO:68) and P4 (5'-gtaccgattatgatctcagttagcctccccatctcccg-3'; SEQ ID  
NO:69) from pcHytk, then digested with BsaI and BsaBI and inserted into pHyg5'C  
following digestion with BsaI (partial) and BsaBI (to completion) to generate pHyg-5'  
30 tk.

Translation of HSV-tk-mediated by HCV 5'UTR (cap-independent, HCV  
nucleotides 38-341) was confirmed by Western blotting analysis of the tk protein. The

vector was designed such that the authentic HCV Core protein translation start site (AUG at position 342 of the viral RNA) serves as the translation initiation site for the tk gene. The functional activity of tk was determined by administration of GCV to the cell culture medium. Continuous GCV application (10-100  $\mu$ M) resulted in complete cell death, whereas untransfected HeLa cells or cells transfected with a similar vector construct expressing Core protein in place of the tk gene remained unaffected by GCV application. To confirm that tk expression was mediated by HCV IRES and not from read-through translation of the hygromycin gene, two control plasmids were generated that were identical to the parental, except for deletion of part of the 5'UTR necessary for IRES function. A reporter cell line was generated by electroporation of HeLa cells with pHyg-5'tk and selected with 250  $\mu$ g/ml hygromycin. Single cell clones of stable HSV-tk expressing cells (HeLa 5'tk cells) were obtained according to standard limited dilution cloning techniques and functionally characterized for GCV-mediated cell killing.

Following clonal expansion, several stable HeLa 5' tk clones were characterized for GCV killing and a particular HeLa 5' tk clone was selected that was completely killed following exposure to 20  $\mu$ M GCV. HSV-tk-negative cells were then added into HeLa 5' tk cells to determine the rate of killing of tk positive cells and recovery of tk negative cells and determined the optimal concentration and exposure time of GCV as well as established conditions with minimized "bystander" effect (toxicity to neighboring cells not expressing the HSV-tk gene). The optimal concentration of GCV was determined to be 10-40  $\mu$ M, applied for 24 to 40 hours after reattachment of the cells after plating. The optimal cell plating density was determined to be  $0.4 \times 10^4$  cells/cm<sup>2</sup>.

Retroviral vectors expressing a neo<sup>R</sup> marker and either a control ribozyme or a library of ribozymes with randomized target recognition sequences were used to stably transduce clonal populations of these reporter cells, which were then subjected to GCV selection (Figure 2). HeLa cells were cultured at 37°C in DMEM (Gibco BRL) supplemented with 10% FBS, L-gln, sodium pyruvate and antibiotics. Retroviral library transduction was performed on clonal HeLa 5' tk cells in sixteen 225cm<sup>2</sup> cell culture flasks using a total of 460 ml of non-concentrated retroviral supernatant (titer  $4 \times 10^5$  CFU/ml determined on HeLa cells, MOI 2). Control retroviral transduction (total

volume 40 ml, titer  $2 \times 10^4$  CFU/ml) was performed in two 225 cm<sup>2</sup> cell culture flasks. 24 hours post transfection, cells were selected with G418 (500 µg/ml) for two weeks.

Following G418 selection, ribozyme gene vector library transduced cells were seeded at a density of  $0.4 \times 10^4$ /cm<sup>2</sup>. This plating density was chosen after optimization studies in order to minimize a potential "bystander" effect. Cells were exposed to GCV at a concentration of 40 µM for 24h or 40h and subsequently cultured under hygromycin selection (250 µg/ml) for 21 days (Figure 3, showing a schematic of the selection system).

Following one round of GCV selection for 40 hours, 25 colonies were obtained in the library transduced cell population versus 8 in the control vector transduced population and 0 in the untransduced population. Library transduced cells exposed to GCV for 24 hours produced 50 resistant colonies (Figure 4A).

The method of Rz gene rescue was performed by PCR amplification of the genomic DNA from the cultured cells, followed by batch recloning of the Rz genes into the pLHPM vector. PCR rescue was performed on genomic DNA, isolated from the selected cells using the QIAamp Blood Kit (Qiagen, Valencia, CA). PCR amplification with primers LHPM-2878 (5'-ggcgggactatggttgctgactaat-3'; SEQ ID NO:70) and 5'fMFT2 (5'-ggttatcacgttcgcctcacacgc-3'; SEQ ID NO:71) were used to amplify a 300 bp fragment containing the ribozyme genes using the PCR protocol of 35 cycles at 94°C for 20 sec, 65°C for 30 sec, and 72°C for 45 sec; terminal extension was performed at 72°C for 7 min. The PCR product, which contained a pool of Rz genes, was then digested with BamHI and MluI and ligated into pLHPM digested with the same enzymes (Ho et al., NAR 24:901-907 (1996)). The resulting plasmid was used to generate enriched ribozyme gene vector library retroviral vector by triple transfection as described previously.

The HeLa Hyg 5' tk cell line was subjected to three additional rounds of transduction, puromycin selection, GCV exposure, and rescue of ribozyme sequences. Enhanced colony formation was seen at each round of selection (Figure 4A). Following the four rounds of selection, the sequences of these rescued ribozyme were determined using primer NL6H6 by standard techniques. The resultant gene sequences of ribozymes that conferred GCV resistance were:

- HVC1:AGCTGGCAGAACTGCaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 47)
- HCV2:TTCGTCAAAGAATTCTaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 48)
- 5 HCV3:GCGATCTAAGAATCAGaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 49)
- HCV4:CTAACTTTAGAACTAaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 50)
- HCV5:CTTGCGCGAGAACATCaccagagaaacacacgttggtacattacctggta  
10 (SEQ ID NO: 51)
- HCV6:TTCTTATTAGAAAGCTaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 52)
- HCV7:TCGCTTAAAGAAGGAAaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 53)
- 15 HCV8:AGCCGAGGAGAATCCCaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 54)
- HCV9:AGCCGCATAGAAGCAGaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 55)
- HCV10:TAATTATTAGAATGCGaccagagaaacacacgttggtacattacctggta  
20 (SEQ ID NO: 56)
- HCV11:AGACCAAAAAGAAGCTTaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 57)
- HCV12:ACAGCCAGAGAAACCGaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 58)
- 25 HCV13:TTAAACGCAGAATACGaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 59)
- HCV14:TATTGGCTAGAACGAAaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 60)
- HCV15:TCAGCCTCAGAACTGCaccagagaaacacacgttggtacattacctggta  
30 (SEQ ID NO: 61)
- HCV16:TTGTTAATAGAACTTaccagagaaacacacgttggtacattacctggta  
(SEQ ID NO: 62)



HCV17:TAACACGTAGAAAGACaccagagaaacacacgttggtacattacctggta

(SEQ ID NO: 63)

HCV18:CTGTCAACAGAACTCGaccagagaaacacacgttggtacattacctggta

(SEQ ID NO: 64)

5 HCV19:ATTCATATAGAATGGAaccagagaaacacacgttggtacattacctggta-

(SEQ ID NO: 65)

Introduction of these individual ribozymes into the HeLa 5' tk selection system again resulted in enhanced colony formation compared to control. Figure 4B shows the number of colonies following transduction with individual ribozymes and Figure 4C shows appearance of colonies following selection with ribozymes 2, 6 and 9. Figure 4D shows the RST region of each of the above ribozymes as well as the corresponding target genes that have been identified (SEQ ID NOS:6 through 24, 72 and 73, respectively).

To verify that the rescued ribozymes act as enzymes and derived phenotypes are not due to anti-sense or aptimer effects, a catalytically inactive version of a ribozyme can be prepared and tested using the functional assay as described above. A triple mutation in the catalytic core of HCV4 and HCV6 were generated by replacing the AAA sequence of loop 2 with a UGC sequence to disable the ribozyme (Figure 2). These changes eliminate the catalytic activity of the ribozyme without altering the target binding sequences. The disabled versions of HCV4 and 6 did not significantly increase the number of GCV resistant colonies over background indicating that the phenotypic changes observed with HCV 4 and 6 are likely due to ribozyme-catalyzed hydrolysis and not aptimer or antisense effects.

### 25 EXAMPLE III

Isolation and characterization of genes that promote HCV-IRES function

This example demonstrates the isolation of full-length nucleic acid molecules that promote IRES function as a translation initiator and determination of the corresponding cDNA and polypeptide sequences.

Since ribozymes recognize their cognate targets by sequence complementarity, the sequence of a ribozyme that causes a phenotype through its catalytic activity

predicts a sequence tag that can be used to clone the target gene. This "Ribozyme Sequence Tag" or RST is 16 bases long consisting of the two target binding arms (helix 1 and 2) and the requisite GUC in the target (Figure 2). The RST can thus be used as a primer for 3' and 5' RACE.

5 Specifically, 3'-RACE can be used to amplify a cDNA fragment which contains the a sequence with homology complementary to the inferred substrate binding site of a ribozyme. mRNAs (2 µg) were extracted from GCV selected HeLa 5' tk cells and a first strand cDNA was synthesized from the mRNA with avian myeloblastosis virus reverse transcriptase and a modified lock-docking oligo(dT) primer (Clontech) according to the  
10 manufacturers recommendations (Marathon cDNA amplification kit, Clontech). The product of this reaction was used as a template in a second strand synthesis reaction. After ligation of the double-stranded cDNAs with the Marathon cDNA adaptor, 3'-RACE amplification was performed using the double-stranded cDNA as a template with a sense primer derived from the sequence of the substrate binding domain of a rescued  
15 ribozyme gene and an adaptor specific primer, AP1 (Clontech). The resulting PCR products were then cloned and sequenced. Since the ribozyme specific primer is incorporated into the PCR product, the exact sequence to which the primer bound during PCR is determined by 5'-RACE using upstream sequences to generate a 5'-RACE anti sense primer.

20 For 5' RACE, mRNAs (2 µg) extracted from GCV selected HeLa 5' tk cells and cDNA was synthesized with avian myeloblastosis virus reverse transcriptase and a modified lock-docking oligo(dT) primer (Clontech) according to the manufacturers recommendations (Marathon cDNA amplification kit, Clontech). After ligation of the double-stranded cDNAs with the Marathon cDNA adaptor, 5'- RACE amplification was  
25 performed using the double-stranded cDNA as a template with a anti-sense primer derived from the sequence of the substrate binding domain of the ribozyme and an adaptor specific primer, AP1 (Clontech). The resulting PCR product was then cloned and sequenced. Since the ribozyme specific primer is incorporated into the PCR product, the exact sequence to which the primer bound during PCR is determined by 3'-  
30 RACE using upstream sequences to generate a sense 3'- RACE primer. In addition, gene-specific primers were generated based on the sequences of progressively amplified 5' products. Finally, the 5'-terminus of the gene was cloned using a SMART

amplification technique (Clontech). PCR-amplified cDNA fragments were cloned into T/A-type PCR cloning vectors (pCR2.1; Invitrogen) and sequenced.

5'-RACE was used to amplify a cDNA fragment which contains a sequence with homology to the inferred substrate binding site of the ribozyme, HCV6. 5'-RACE  
5 amplification was performed using the double-stranded cDNA as a template with a anti-sense primer derived from the sequence of the substrate binding domain of HCV6 (5'-ttcttattgacnagct-3'; SEQ ID NO:74). Identified was a 580 bp 5' fragment sharing homology to the rat eIF2B gamma subunit gene (eIF2B $\gamma$ ) (GenBank accession number U38253). To obtain the terminal 5' nucleotides of the mRNA, SMART PCR  
10 amplification was performed with oligo(dT) as a primer for reverse transcription.

To identify RNAs which can be specifically cleaved by a particular ribozyme, a 3'-RACE technique was developed which preferentially amplifies the 3' product of an *in vitro* cleavage reaction. Approximately 1  $\mu$ g of mRNA was incubated with 300 ng of a specific ribozyme in conditions which support ribozyme cleavage (10 mM Tris HCl, 12  
15 mM MgCl<sub>2</sub>, 37°C). cDNA was then synthesized using an Superscript reverse transcriptase (Life Technologies), and an anchored oligo-dT primer for reverse transcription followed by second strand synthesis using a SMART primer (Clontech). This resulted in the addition of SMART primer sequences to the 5' end of the cDNAs including any cDNAs generated from 3' ribozyme cleavage products. The cleavage  
20 products were then preferentially PCR amplified by using a 5' primer which includes sequences found in the SMART oligo as well as bases which include the NGUC ribozyme cleavage site. Compared with the 5' end obtained from the 5'-RACE amplification, an additional 88 nucleotides of 5' sequence was obtained.

Based on this sequence information, the entire human eIF2B $\gamma$  gene was cloned  
25 (Figure 5A), which encodes a protein of 452 amino acids, with 91% identity to the rat protein (Figure 5B). The putative RzHCV6 binding site contains one mismatch and two G-U base pairs in helix 1 of the Rz binding domains (Figure 5C).

Target binding sequences, which are inferred from the sequences of rescued ribozymes, can also be used to query nucleotide databases. Using a BLAST search, the  
30 binding sequence of HCV4 partially matched a sequence within the gamma subunit of human eIF2 (GenBank accession number L19161). As this is a known gene, no further cloning was necessary.

Ribozymes can also be used to identify genes by radiolabeled oligonucleotide screening of cDNA libraries. <sup>32</sup>P labeled oligonucleotides which correspond to the inferred cleavage sites of rescued ribozymes HCV8 have been used to screen cDNA libraries by means of standard filter hybridization methods.

5

#### EXAMPLE IV

##### Validation of gene function

This example shows that knockdown of eIF2By or eIF2γ mRNA by several  
10 different gene targeted ribozymes confers GCV resistance in HeLa 5' tk cells, confirming that these genes are regulators of IRES mediated translation.

To confirm eIF2By as a target gene involved in GCV resistance and HCV core protein expression, five validation ribozymes were designed (TVRz1-5) that were directed against additional GUC sites in the human eIF2By mRNA. The nucleotide  
15 sequences of the TSTs identified by these validation ribozymes are shown below in Table 1 along with other eIF2By TST sequences (SEQ ID NOS:75 through 95, respectively). These validation ribozymes were cloned into retroviral vectors for transduction of HeLa 5' tk cells. TVRz2-5 conferred greater than 10-fold increase in GCV-resistant colonies compared with the control. The results are shown in Figures 6A  
20 and B.

Table 1

GUC sites for human eIF2By

<u>position</u>	<u>Sequence</u>	<u>TVRz No.</u>
80	CAGC GGUC UGACCCGG	
81	AAGA AGUC AUUGUGGU	1
82	CCC GGUC AAAAGGGG	
83	AGCU GGUC AUUAGGG	2
84	CACG GGUC UUGUGGAU	3
85	AAUG GGUC AAUAACUU	4
86	CUGA AGUC CUUAGAU	

87	UGCC UGUC GAGGAGAC
981	GACU UGUC CAGAUAC
1006	GCUA UGUC CACAUCAU
1007	UUGC UGUC UGCUCUCU
1008	UCUC UGUC CAGAAGAA
1009	CACC AGUC CAUUCGUC
1010	CAUU CGUC AGCCCAGA
1011	AGAU UGUC AGCAAACA
1012	GAGA AGUC AUCCAUA
1013	GCUC AGUC AUUGGCUC
1014	AUCC UGUC UCAUAAAA
1015	ACUC AGUC ACUGUGGA
1016	GCAG UGUC AUCUGCAA
1017	AGCA AGUC AGACUCCU

5

Validation ribozymes were also constructed against four potential cleavage sites in the eIF2 $\gamma$  mRNA (TVRz6-9). The nucleotide sequences of the TSTs identified by these validation ribozymes are shown below in Table 2 along with other eIF2 $\gamma$  TST sequences (SEQ ID NOS:96 through 111). Transduction of HeLa 5' tk cells with retroviral, vectors expressing these ribozymes induced greater than 9-fold increase in the number of GCV resistant colonies relative to the control (Figure 7A).

Table 2GUC sites for human eIF2 $\gamma$ 

position	Sequence	TVRz No.
50	UUCG CGUC AGGAUCUC	
143	AAUU GGUC AUGUAGCU	6
169	CCAC AGUC GUCAAAGC	
172	CAGU CGUC AAAGCUAU	7
199	AUAC UGUC AGGUUCAA	8
379	AAUU AGUC AGACAUGU	9
404	UGAC UGUC CUGGCCAC	
593	AGAA AGUC AGGCUAAA	
631	CAUU UGUC CAAGGUAC	
790	UUGA UGUC AACAAACC	
1004	AGGC GGUC UUAUUGGA	
1083	GUGC AGUC GGAGCUUU	
1194	AAGC UGUC UAAGAAUG	
1233	UCCC UGUC AACAGGAG	
1258	GUGC UGUC AAGGCCGA	
1370	UUGG GGUC AGAUAAGA	

5 Two other known genes were identified as TSTs from the library screen. HCV2 corresponds to human proteasome alpha subunit 1 PSMA1. HCV9 corresponds to human proteasome alpha subunit 7 PSMA7. The TST nucleotide sequences for the human PSMA1 and PSMA7 cellular regulators for target validation ribozymes are shown below in Table 3 and Table 4 respectively (SEQ ID NOS:112 through 139).

Table 3

GUC sites for human PSMA1

<u>position</u>	<u>Sequence</u>
133	AUGA UGUC ACUGUUUG
215	AGUU GGUC UGAAAUCA
371	UAUG CGUC AGGAGUGU
420	CCUG UGUC UCGUCUUG
425	GUCU CGUC UUGUAUCU
497	UGUU GGUC UCCUUAUU
548	AACC UGUC CAUCUGCU
633	CAUA UGUC UGAAUUUA
677	ACAU GGUC UGCGUGCC
792	GAUG UGUC UCCAUUCC
809	GGAA GGUC UUGAAGAA
909	AGCC AGUC UAUUAUUG
1028	AAUC AGUC CAGAUGUG
1099	AAAG GGUC UGUAAUAAU
1165	UAGG UGUC UUUGUGGU

Table 4

GUC sites for human PSMA7

<u>position</u>	<u>Sequence</u>
49	UCAC CGUC UUCUCGCC
100	AGGC CGUC AAGAAGGG
168	AAGA AGUC AGUGGCCA
229	ACAA CGUC UGCAUGGC
271	GGAU AGUC AUCAACAG
328	ACCC GGUC ACUGUGGA
362	CGCC AGUC UGAAGCAG
522	GCCA AGUC AGUGCGCG
624	GUUC AGUC AGGUGGCA
652	UUGC UGUC AUGAGGCG
782	AAAA UGUC UUUGCUUG
824	GAUG AGUC UCGAUGUG
868	UGAG UGUC CUACAAUA

- 5 To ensure that the above-described validation ribozymes did not interfere with the transport or metabolism of GCV, their effects were evaluated on IRES-mediated HCV core protein translation from the cassette within the retroviral vector (pLHPM). Upon construction of the retroviral vector pLHPM, a cassette translating the HCV Core protein via the HCV IRES was placed into the 3' part of the LTR transcript. Thereby,
- 10 cells transduced with the library or single Rz candidates could be analyzed for the HCV Core protein expression by Western blotting. Western blotting was performed on protein lysates using an enhanced chemiluminescent blotting detection kit (Novex), and anti-HCV core monoclonal antibody (6C7 provided by Harry Greenberg, Stanford University). Blots were exposed to film and band intensities measured using a
- 15 phosphoimager and ImageQuan software. After establishment of a linear relationship of the amount of Core protein and the measured signal (data not shown), GCV resistant colonies derived from the single Rz transduction were analyzed for HCV Core protein. Band volumes of HCV Core protein in Western blotting were analyzed by densitometry,



normalized towards beta-actin and expressed as percentage relative to the control (RzBR1)

A decrease in Core protein was observed in several candidates (range 16 to 59%, Figure 8A), with RzHCV8 showing an exceptional decrease in Core protein expression with 95% reduction compared to control Rz transduced cells after GCV selection (Figure 8B). A reduction in core protein expression to 52% and 42% was observed in GCV-resistant cells derived from RzHCV6 and RzHCV4 transduced cells, respectively, compared to control RzBR1 transduced cells (Figures 8A and B). Figure 8A is a western blot where lane 1 shows protein levels for the HeLa 5' tk parental cells and lanes 2-10 shows the protein levels of cells transduced with respectively BR1, HCV control ribozyme, and HCV8, HCV2, HCV6, HCV5, HCV4, HCV9, and HCV1. Figure 8B shows the quantitation of the western blot in figure 8A. Target validation ribozymes 2 and 4 showed 26% and 35% reduction, respectively (Figure 6B). Target validation ribozymes 6-9 showed approximately 70% reduction in core protein (Figure 7B).

To assure that the observed reductions in Core protein were not caused by reduced levels of RNA transcript, Northern blotting analysis was performed on polyA-RNA extracted from stable Rz expressors after GCV selection. Total RNA (15 µg per lane) extracted from cultures at 80% confluency was separated by formaldehyde/1% agarose gel electrophoresis and transferred to nylon membrane. The RNAs were probed for different parts of the two vector derived transcripts expressed in these cells: probes were directed against the tk part of the bicistronic transcript or against the Core portion of the retroviral vector transcript respectively. <sup>32</sup>P dCTP labeled probes were hybridized to the membrane in Quickhyb solution (Stratagene) at 65°C. After washing in 2xSSC/0.1%SDS and exposure to film, quantitation of signal was achieved by phosphimager analysis and computer-assisted denistometry as for the Western procedure. Levels of LTR-Core (4.8 kb) and HSV-tk (2.8 kb) transcripts were normalized to signals derived from GAPDH and expressed as percentage of the signal obtained from control Rz transduced 5' Itk cells.

Whereas for RzHCV8 a 20% reduction of Core transcript was detected, all other candidates tested showed a relative Core RNA level of equal or higher compared with control Rz transduced cells. No difference was detected for the expression of GAPDH or tk transcript between the GCV resistant cells derived from single Rz transduction.

Comparable levels of tk-transcript between parental cells, 5'-tk and ribozyme expressing cells do not explain the gain of resistance to GCV observed in the ribozyme expressing colonies. All preparations of ribozyme vectors had comparable titer. However, to eliminate differences in expression of the retroviral transcript (harboring the 5' UTR  
5 Core transcript) between different Rz vector transduced cells, the ratio between expressed Core protein and RNA transcript levels as an indicator of HCV IRES translational activity was used.

The ratio of core protein/RNA transcript in GCV resistant colonies indicates a decrease in IRES activity for several single ribozymes compared with control Rz BR1  
10 transduced, GCV selected cells. The quantitation of these protein levels are shown in Figure 9. A reduction of HCV IRES-dependent translatability of 83% and 76% was observed for HCV6 and HCV4, respectively (Figure 9). In contrast, no changes in cell growth and cap-dependent translation of cellular proteins (GAPDH, b-actin, c-myc) in GCV- and hygromycin-resistant cells derived from transduction with the functional  
15 ribozyme vectors or "validation" ribozymes compared with control Rz transduced cells were observed.

Throughout this application various publications have been referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of  
20 the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention.  
25 Accordingly, the invention is limited only by the following claims.

CLAIMS

1. A substantially pure nucleic acid comprising a nucleotide sequence greater than about 80% identical to SEQ ID NO:1, or a complementary sequence thereof.  
5
2. The substantially pure nucleic acid of claim 1 wherein said nucleotide sequence comprises SEQ ID NO:1.
3. A substantially pure nucleic acid comprising at least 16 contiguous nucleotides  
10 in length selected from SEQ ID NO:1 or selected from a sequence of nucleotides having greater than about 80% identity to SEQ ID NO: 1, or a complementary sequence thereof.
4. The substantially pure nucleic acid of claim 4, wherein said sequence is selected  
15 from SEQ ID NO:1.
5. The substantially pure nucleic acid of claim 4, wherein said nucleotide sequence comprises eIF2By codons selected from the group consisting of 57, 71, 81, 92, 105, 137, 139, 196, 211, 219, 245, 248, 258, 269, 270, 274, 285, 287, 290, 332, 338, 339, 340,  
20 342, 347, 348, 352, 354, 360, 361, 364, 380, 382, 399, 403, 405, 410, 415, 417, 420, 422, and 439.
6. A substantially pure nucleic acid comprising at least 18 contiguous nucleotides in length selected from SEQ ID NO:1 or selected from a sequence of nucleotides having  
25 greater than about 80% identity to SEQ ID NO: 1, or a complementary sequence thereof.
7. The substantially pure nucleic acid of claim 6, wherein said sequence is selected from SEQ ID NO:1.

8. The substantially pure nucleic acid of claim 6, wherein said nucleotide sequence comprises eIF2B $\gamma$  codons selected from the group consisting of 57, 71, 81, 92, 105, 137, 139, 196, 211, 219, 245, 248, 258, 269, 270, 274, 285, 287, 290, 332, 338, 339, 340, 342, 347, 348, 352, 354, 360, 361, 364, 380, 382, 399, 403, 405, 410, 415, 417, 420, 422, and 439.

9. A ribozyme that hybridizes to and cleaves a cellular regulator of hepatitis C virus replication, said ribozyme comprising a target recognition sequence that selectively hybridizes and cleaves RNA encoded by SEQ ID NO:1 or encoded by a sequence of nucleotides selected from the group consisting of GenBank accession numbers: AA703831, AI800599.1, AI632282.1, W58368, W58049, A1660531.1, A1798535.1, AI143649, AJ040925, AA102365, AA991764, AA587233, AA580025, A1637675.1, W22190, A1937500.1, T78051, R38705, T90276, T82858, AA507077, Z41323, Z45650, AA649500, T75394, A1700227.1, F01614, A1359536, AA582198, F00408, AA322492, Z24924, F31617.1, AA102364, AA905387, C21034, F05355, AA933569, F34596.1, A1970443.1, AA308943, AA323715, R61886, AA130818, AA311999, AA076401, and AA703831.

10. The ribozyme of claim 9, wherein said ribozyme target recognition sequence comprises the sequence N<sub>8</sub>-AGAA- N<sub>4</sub>.

11. The ribozyme of claim 9, wherein between 10-12 nucleotides of segments N<sub>8</sub> and N<sub>5</sub> taken together are complementary to the RNA target.

25

12. The ribozyme of claim 9, wherein said ribozyme target recognition sequence selectively hybridizes to and cleaves RNA encoded by SEQ ID NO:1.

13. The ribozyme of claim 12, wherein said target recognition sequence comprises an RST complementary to a fragment of SEQ ID NO:1 and having substantially the nucleotide sequence N<sub>8</sub>-AGAA-N<sub>4</sub>.

30

14. The ribozyme of claim 13, wherein between 10-12 nucleotides of the segment N<sub>8</sub> and N<sub>5</sub> taken together are complementary to the RNA target.

15. The ribozyme of claim 12, further comprising the sequence 5'-  
5 UUCUUAUUAGAAAGCU-3' (SEQ ID NO:16).

16. A ribozyme that hybridizes to and cleaves a cellular regulator of hepatitis C virus replication, said ribozyme comprising a target recognition sequence selected from the group consisting of SEQ ID NOS: 6-24, 47-65, 72 and 73.

10

17. The ribozyme of claim 16, wherein said ribozyme target recognition sequence is selected from the group consisting of SEQ ID NOS: 47-65.

18. A substantially pure nucleic acid encoding a cellular regulator of hepatitis C  
15 virus replication, comprising a nucleotide sequence selected from the group consisting of SEQ ID NOS: 26-34, 36, and 38-42.

19. A substantially pure polypeptide comprising an amino acid sequence greater than about 91% identical to SEQ ID NO:2.

20

20. The substantially pure polypeptide of claim 19 comprising the amino acid sequence shown in SEQ ID NO:2.

21. A method of identifying a compound that modulates the activity of a viral  
25 cellular regulator comprising,

a) obtaining a sample comprising a protein or nucleic acid element acted upon by a cellular regulator, said nucleic acid element optionally linked to a reporter gene sequence;

b) contacting the sample with the cellular regulator and a test compound  
30 under conditions that allow viral replication mediated by the protein or nucleic acid element to occur or allow expression of the nucleic acid element or its linked reporter gene to occur; and

c) measuring the amount of viral replication or expression wherein an increase or decrease in the amount of replication or expression in the presence of the test compound compared to the absence of the test compound indicates that the compound has cellular regulator modulatory activity.

5

22. The method of claim 21, wherein said test compound decreases the amount of replication or expression of said nucleic acid element or associated reporter gene, thereby exhibiting cellular regulator inhibitory activity.

10 23. The method of claim 21, wherein said test compound increases the amount of replication or expression of said nucleic acid element or associated reporter gene, thereby exhibiting cellular regulator enhancing activity.

24. The method of claim 21, wherein said cellular regulator is selected from the  
15 group consisting of eIF2B $\gamma$  (SEQ ID NOS:1, 2, 16 and 35), eIF2 $\gamma$  (SEQ ID NOS:6 and 25), human proteasome alpha subunit PMSA1 (SEQ ID NOS:18 and 37) and human proteasome alpha subunit PMSA7 (SEQ ID NOS:24 and 43).

25. The method of claim 21, wherein said viral nucleic acid element is from  
20 hepatitis C virus.

26. The method of claim 21, wherein said viral nucleic acid element is an internal ribosome entry site.

27. A method of identifying a compound that modulates the activity of a viral cellular regulator comprising, a) contacting a sample with a cellular regulator and a test compound, said sample comprising a protein or nucleic acid element acted upon by a cellular regulator, said nucleic acid element optionally linked to a reporter gene sequence the sample, said contacting under conditions that allow viral replication mediated by the protein or nucleic acid element to occur or allow expression of the nucleic acid element or its linked reporter gene to occur; and b) measuring the amount of viral replication or expression wherein an increase or decrease in the amount of replication or expression in the presence of the test compound compared to the absence of the test compound indicates that the compound has cellular regulator modulatory activity.
28. The method of claim 27, wherein said test compound decreases the amount of replication or expression of said nucleic acid element or associated reporter gene, thereby exhibiting cellular regulator inhibitory activity.
29. The method of claim 27, wherein said test compound increases the amount of replication or expression of said nucleic acid element or associated reporter gene, thereby exhibiting cellular regulator enhancing activity.
30. The method of claim 27, wherein said cellular regulator is selected from the group consisting of eIF2B $\gamma$  (SEQ ID NOS:1, 2, 16 and 35), eIF2 $\gamma$  (SEQ ID NOS:6 and 25), human proteasome alpha subunit PMSA1 (SEQ ID NOS:18 and 37) and human proteasome alpha subunit PMSA7 (SEQ ID NOS:24 and 43).
31. The method of claim 27, wherein said viral nucleic acid element is from hepatitis C virus.
32. The method of claim 27, wherein said viral nucleic acid element is an internal ribosome entry site.

33. A method of identifying a ribozyme reactive with a cellular regulator of virus replication or expression, comprising: (a) introducing a randomized ribozyme gene vector library into a population of cells expressing a negative selection marker gene operatively linked to a viral nucleic acid element acted on by a cellular regulator of virus replication or expression; (b) subjecting said population of cells to negative selection; and (c) recovering one or more ribozymes from viable cells following said negative selection.

34. The method of claim 33, wherein said ribozyme gene vector library comprises hairpin ribozymes.

35. The method of claim 33, wherein said negative selection marker comprises thymidine kinase, cytosine deaminase or diphtheria toxin.

36. The method of claim 33, wherein said negative selection further comprises contacting the cells with a compound selected from the group consisting of ganciclovir, FIAU or 5-fluorocytosine.

37. The method of claim 33, wherein said viral nucleic acid element is from hepatitis C virus.

38. The method of claim 33, wherein said viral nucleic acid element is an internal ribosome entry site.

39. A method of identifying a cellular regulator of virus replication or expression, comprising: (a) introducing a randomized ribozyme gene vector library into a population of cells expressing a negative selection marker gene operatively linked to a viral nucleic acid element acted on by a cellular regulator of virus replication or expression; (b) subjecting said population of cells to negative selection; (c) recovering one or more ribozymes from viable cells following said negative selection; and (d) sequencing the target recognition sequence of said recovered ribozyme to identify the nucleic acid encoding said cellular regulator.



40. The method of claim 39, wherein said ribozyme gene vector library comprises hairpin ribozymes.
- 5 41. The method of claim 39, wherein said negative selection marker comprises thymidine kinase, cytosine deaminase or diphtheria toxin.
42. The method of claim 39, wherein said step of negative selecting further includes contacting the cells with a compound selected from the group consisting of ganciclovir,  
10 FIAU or 5-fluorocytosine.
43. The method of claim 39, wherein said viral nucleic acid element is from hepatitis C virus.
- 15 44. The method of claim 39, wherein said viral nucleic acid element is an internal ribosome entry site.
45. A method of treating an HCV infection by inhibiting the activity of a cellular regulator involved in HCV replication, comprising administering a ribozyme selectively  
20 reactive with an RNA encoding the cellular regulator, administering a nucleic acid vector encoding a ribozyme selectively reactive with an RNA encoding the cellular regulator, administering a compound that inhibits the activity of the cellular regulator or administering an antisense nucleic acid that inhibits transcription or translation of the cellular regulator.
- 25 46. The method of claim 45 wherein said cellular regulator is a subunit of a eukaryotic translation initiation factor or a proteasome.
47. The method of claim 46, wherein said eukaryotic translation initiation factor is  
30 eIF2 or eIF2B.
48. The method of claim 47, wherein said subunit comprises eIF2 $\gamma$  or eIF2B $\gamma$ .

49. The method of claim 46, wherein said subunit comprises substantially the same amino acid sequence as SEQ ID NO:2.
- 5 50. The method of claim 46, wherein said subunit is proteasome alpha subunit.
51. The method of claim 45, wherein said ribozyme comprises a target recognition sequence selected from the group consisting of 5'-CUAACUUUAGAAACUA-3', 5'-UUCUUAUUAGAAAGCU-3', 5'-UUCGUCAAAGAAUUCU-3', or 5'-
- 10 AGCCGCAUAGAAGCAG-3' (SEQ ID NOS: 6, 16, 18 and 24, respectively).
52. The method of claim 45, wherein said administered ribozyme is a hammerhead ribozyme.
- 15 53. The method of claim 45, wherein said ribozyme encoded by said vector is a hairpin ribozyme.
54. The method of claim 45, wherein a nucleic acid vector encoding a ribozyme selectively reactive with an RNA encoding the cellular regulator is administered.
- 20 54. The method of claim 54 wherein said ribozyme comprises the target recognition sequence of claim 9.

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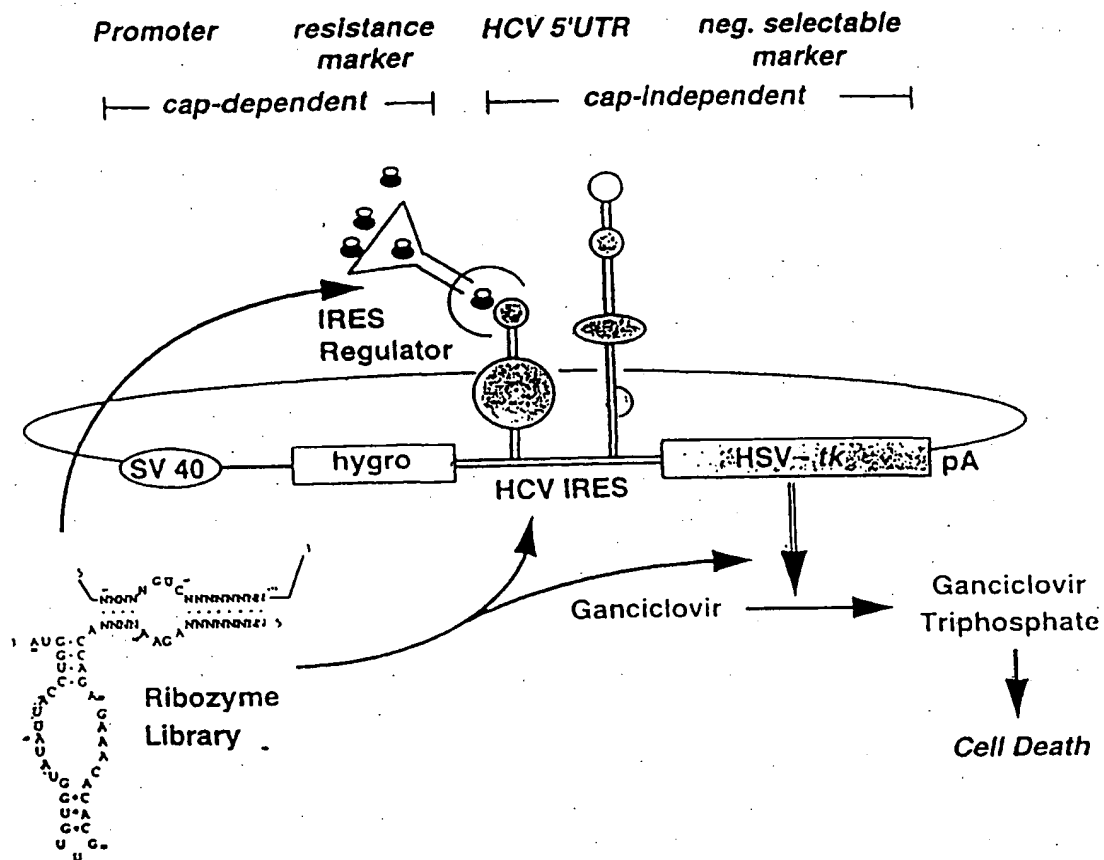


FIGURE 1



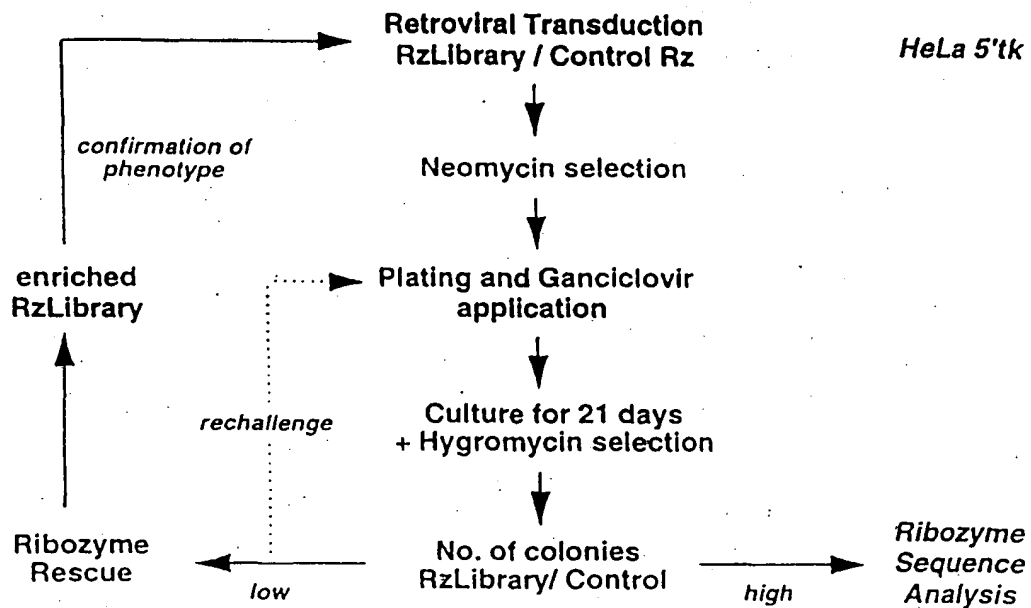


FIGURE 3

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a

round I, II, IV

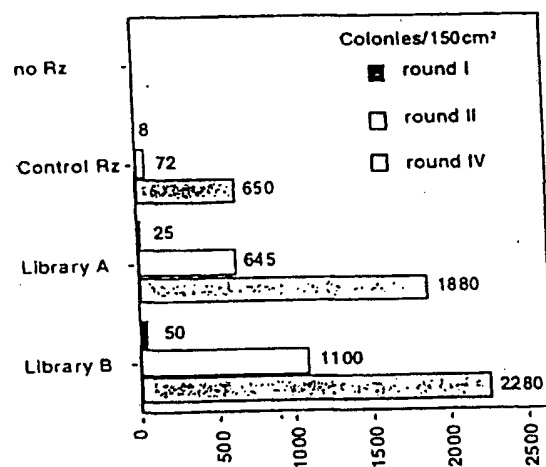


FIGURE 4A

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b

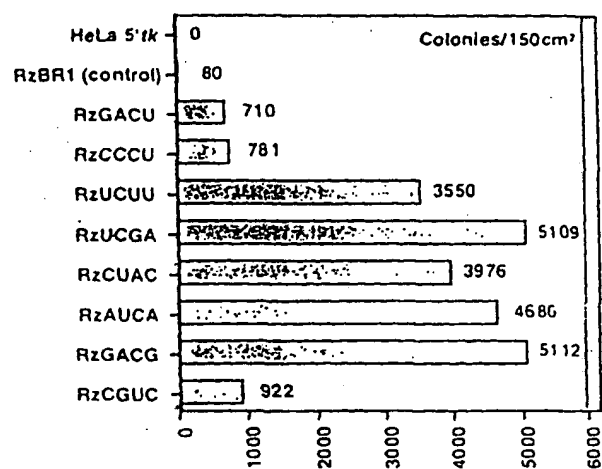
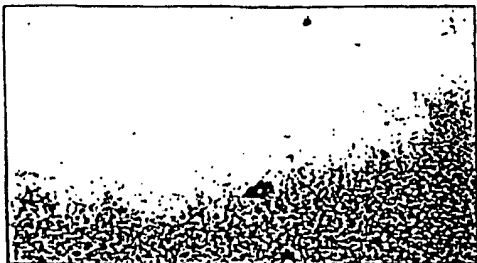
single Rz candidates  
(derived from round IV)

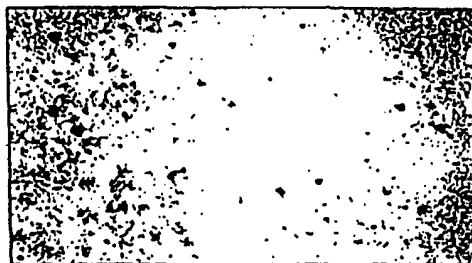
FIGURE 4B

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A. Control Rz (RzBR1)



C. RzUCUU



B. RzUCGA



D. RzGACG

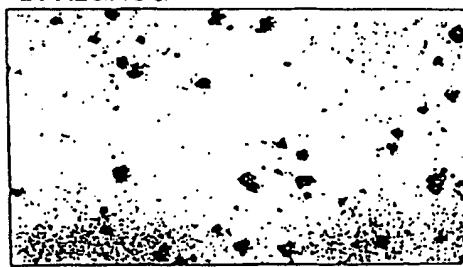


FIGURE 4C



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C

Ribozyme	Sequence		Target Gene	
	helix 1	helix 2	(match)	
Rz Pool	5' - NNNNNNNN	agaa NNNN -3'		
Library Exp. A				
RzAUCA	5' - CUAACUUU	agaa ACUA -3'	human eIF2γ	(13/16)
RzGCGU	5' - UAAUUAUU	agaa UGCG -3'		
RzGACU	5' - GCGAUCUA	agaa UCAG -3'		
RzUUCG	5' - AGACCAAA	agaa GCUU -3'		
RzGCCA	5' - ACAGCCAG	agaa ACCG -3'		
RzGCAU	5' - UUAACGC	agaa UACG -3'		
RzAAGC	5' - UAUUGGCU	agaa CGAA -3'		
RzCGUC	5' - UCAGCCUC	agaa CUGC -3'		
RzCGUC	5' - AGCUGGC	agaa CUGC -3'		
RzUUCA	5' - UUGUAAU	agaa ACUU -3'		
RzUCGA	5' - UUCUAAU	agaa AGCU -3'	human eIF2Bγ	(19/22)
Library Exp. B				
RzAAGG	5' - UCGCUUAA	agaa GGAA -3'		
RzUCUU	5' - UUCGUCAA	agaa UUCU -3'	human proteasome alpha subunit, PSMA1	(14/16)
RzCAGA	5' - UAACACGU	agaa AGAC -3'		
RzCCCU	5' - AGCCGAGG	agaa UCCC -3'		
RzGCUC	5' - CUGUCAAC	agaa CUCG -3'		
RzAGGU	5' - AUUCAUAU	agaa UGGA -3'		
RzCUAC	5' - CUUGCGCG	agaa CAUC -3'		
Control Rz				
RzGACG	5' - AGCCGCAU	agaa GCAG -3'	human proteasome alpha subunit, PSMA7	(15/16)
RzBR1	5' - AGGUUGGG	agaa GCGA -3'	human hepatitis B virus	
RzGCCA	5' - AUUGCCAG	agaa ACCG -3'	human hepatitis C virus	

FIGURE 4D

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1 GGAGATCGCTGGGAGCGGTTGCCCGTGC CGGGAGCTGAGTTA 42  
TAGCTGTGACTTCTGCCCTGCCAGGCCGCACACAGCGGTC TGACCCGGTTTGGTTTGTA 102  
ATGGAATTTCAAGCAGTAGTGATGCCAGTAGGTGGAGGATCTCGGATGACAGACCTAACT 162  
1 H E F Q A V V H A V G G G S R M T D L T  
TCCAGCATTCCCAAACCTCTGCTTCCAGTTGGGAACAAACCTTTAATTTGGTACCCATTG 222  
21 S S I P K P L L P V G N K P L I W Y P L  
AACCTGCTTGAGCGTGTGGATTGAAGAAGTCATTGTGGTTACAACCAGGGATGTTCAA 282  
41 N L L E R V G F E E V I V V T T R D V Q  
AAGGCTCTATGTCAGAAATCAAGATGAAAATGAAGCCAGATATTGTGTGATTCTCGAT 342  
61 K A L C A E F K M K M K P D I V C I P D  
GACGCTGACATGGAACTGCAGATTCTTTGCCGTACATATATCCAAAACCTTAAGACAGAT 402  
81 D A D M G T A D S L R Y I Y P K L K T D  
CTGCTGGTCTGACCTGTGATCTGATAACAGACGTTGCCCTTACATGAGGTTGTGGACCTG 462  
101 V L V L S C D L I T D V A L H E V V D L  
TTTAGAGCTTATGATGCATCACTTGTCTATGTTGATGAGAAAAGGCCAAGATAGCATAGAA 522  
121 F R A Y D A S L A M L M R K G Q D S I E  
CCTGTTCCCGGTCAAAGGGGAAAAAAGCAGTGGAGCAGCGTGACTTCATTGGAGTG 582  
141 P V P G Q K G K K A V E Q R D F I G V  
GACAGCACAGGAAAGAGGCTGCTCTTCATGGCTAATGAAGCAGACTTGGATGAAGAGCTG 642  
161 D S T G K R L L F H A N E A D L D E E L  
GTCATTAAGGGATCCATCCTACAGAAGCATCCTAGAATACGTTTCCACACGGGCTTGTG 702  
181 V I K G S I L Q K H P R I R F H T G L V  
GATGCCCACCTCTACTGTTTGA AAAATACATCGTGGATTTCCTAATGGA AAAATGGGTCA 762  
201 D A H L Y C L K K Y I V D F L M E N G S  
ATAACTTCTATCCGAGTGAACCTGATCCATATTTAGTGAGAAAACAGTTTTCCTCAGCT 822  
221 I T T S I R S E L I P Y L V R K Q F S S A  
TCCTCACAAACAGGACAGAAGAAAAAGAGGAGGATCTAAAGAAAAAGGAGCTGAAGTCC 882  
241 S S Q Q G Q E E K E E D L K K K E L K S  
TTAGATATCTACAGTTTATAAAGAAGCCAATACACTGAACCTGGCTCCCTATGATGCC 942  
261 L D I Y S F I K E A N T L N L A P Y D A  
TGCTGGAATGCCTGTGAGGAGACAGGTGGGAAGACTTGTCAGATCAGAGGTGGCGTGC 1002  
281 C W N A C R G D R W E D L S R S Q V R C  
TATGTCCACATCATGAAAGAGGGGCTCTGCTCTCGAGTGAGCACACTGGGACTCTACATG 1062  
301 Y V H I M K E G L C S R V S T L G L Y H  
GAAGCAAACAGACAGGTGCCCAAATGTGCTGCTCTGCTGTCAGAAAGAACCACTGTC 1122  
321 E A N R Q V P K L L S A L C P E E P P V  
CATTGCTCAGCCCAGATTGTGAGCAACACCTGGTTGGAGTTGACAGCCTCATGGGCCA 1182  
341 H S S A Q I V S K H L V G V D S L I G P  
GAGACACAGATTGGAGAGAAGTCATCCATTAAAGCGCTCAGTCATTGGCTCATCTGTCTC 1242  
361 E T Q I G E K S S I K R S V I G S S C L  
ATAAAGATAGAGTGACTATTACCAATTGCCCTTCTCATGAACTCAGTCACTGTGGAGGAA 1302  
381 I K D R V T I T N C L L M N S V T V E E  
GGAAGCAATATCCAAGGCAGTGTCTGCAACAATGCTGTGATCGAGAAGGGTGCAGAC 1362  
401 G S N I Q G S V I C N N A V I E K G A D  
ATCAAGGACTGCTTGAATTGGAAGTGGCCAGAGGATTGAAGCCAAAGCTAAACGAGTGAAT 1422  
421 I K D C L I G S G Q R I E A K A K R V N  
GAGGTGATCGTGGGAATGACCAGCTCATGGAGATCTGAGTTCTGAGCAAGTCAGACTCC 1482  
441 E V I V G N D Q L M E I  
TTCTTTTGGCCTCCAAAGCCACAGATGTTGGCCGGCCACCTGTTTAACTCTGTATTTA 1542  
TTTCCCAATAAAGAGGGCTTCCAAAGTA 1602

FIGURE 5A

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human eIF2B $\gamma$	MEFQAVVHAVGGGSRMTDLTSSIPKPLLPGVGNKPLIWYPLNLLERVGFEE	50
rat eIF2B $\gamma$	MEFQAVVHAVGGGSRMTDLTSSIPKPLLPGVGNKPLIWYPLNLLERVGFEE	50
human eIF2B $\gamma$	VIWVTTTRDVQKALCAEFKMKKLPDIVCIPDDADMGTADSLRYIYPKLKTD	100
rat eIF2B $\gamma$	VIWVTTKDVQKALCAEFKMKKLPDIVCIPDEADMGTADSLRIIYPKLKTID	100
human eIF2B $\gamma$	VLVLSCDLITDVALHEVVDLFRAYDASLAMLHRKGQDSIEPVPGQKQKXX	150
rat eIF2B $\gamma$	VLVLGCDLITDVALHEVVDLFRAYDASLAMLHRKGQESTEPVPGQKQKXX	150
human eIF2B $\gamma$	AVEQRDFIGVDSGKRLLFMANEADLDEELVIKGSILQKHPRIRFHTGLV	200
rat eIF2B $\gamma$	TVEQRDFIGVDSGKRLLFMANEADLDEELVIKGSILQKHPRIRFHTGLV	200
human eIF2B $\gamma$	DAHLYCLKKYIVDFLMENGSIITSIRSELIPLYVRKQFSSASSQQQEEKE	250
rat eIF2B $\gamma$	DAHLYCLKKYVVDLFMENKSIITSIRSELIPLYVRKQFSSASSQQQEDKE	250
human eIF2B $\gamma$	EDLKKKELKSLDIYSFIEANTLNLA PYDACWNACRGDRWEDLSRSQVRC	300
rat eIF2B $\gamma$	EDLKKKEPKSLDIYSFIKKONTLTLA PYDACWNAFRDKWEDLSRSQVRC	300
human eIF2B $\gamma$	YVHIMKEGLCSRSTLGLYMEANRQVPKLLSALCPEEPVHSSAQIVSKH	350
rat eIF2B $\gamma$	YVHIMKEGLCSRSTLGLYMEANRQVPKLLSVLCPEESMIHPSAQIANNH	350
human eIF2B $\gamma$	LVGVDSLIGPETQIGEKSSIKRSVIGSSCLIKDRVTITNCLLMNSVTVEE	400
rat eIF2B $\gamma$	LIGADSLIGSDTQVGEKSSIKRSVIGSSCVIRDRVTVTNCLLMNSVTGCE	400
human eIF2B $\gamma$	GSNIQGSVICNNAVIEKGADIKDCLIGSGQRIEAKAKRVNEVIVGNDQLM	450
rat eIF2B $\gamma$	GSSIHGSVIFNNAVVEAGAEIRDCLIGSGQRIEAKAKRMNEVIVGNDQLM	450
human eIF2B $\gamma$	EI*... 452	
rat eIF2B $\gamma$	EI*... 452	

FIGURE 5B

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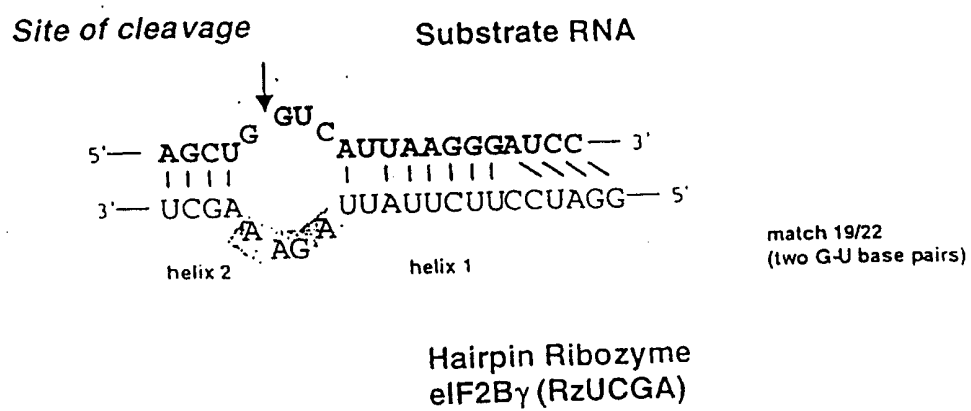


FIGURE 5C

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	Control	HCV6	TV1	TV2	TV3	TV4	TV5
	1	2	3	4	5	6	7
Colonies/150cm <sup>2</sup>	58	960	606	682	976	880	560
Fold enrichment	-	16.6	10.5	11.8	16.8	15.2	9.7
Relative eIF2 $\beta$ RNA expression (% of control)	100	78	n.d	85	103	81	80
Relative Core RNA expression (% of control)	100	256	144	202	166	189	131

FIGURE 6A

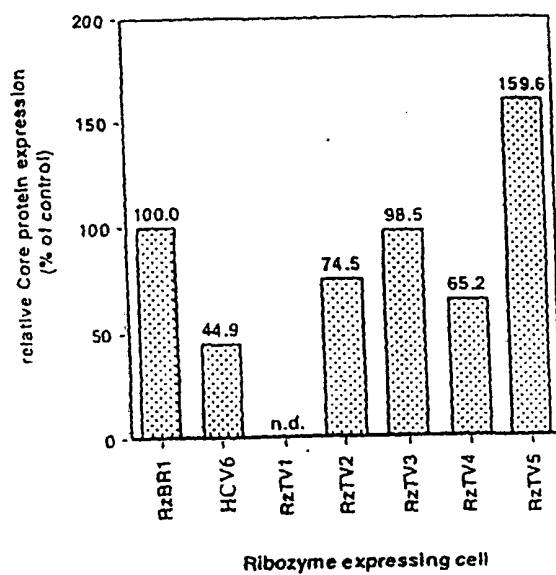


FIGURE 6B

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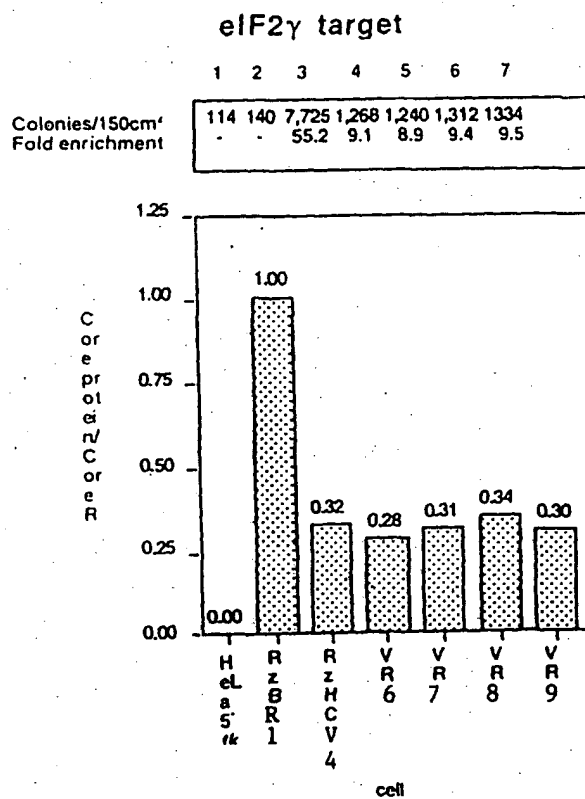


FIGURE 7

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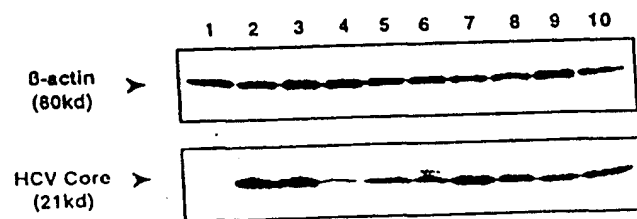


FIGURE 8A



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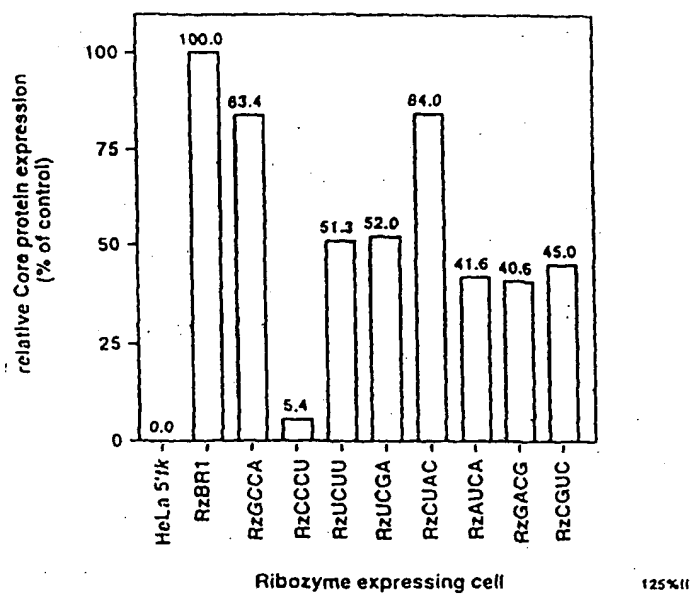
*b*

FIGURE 8B

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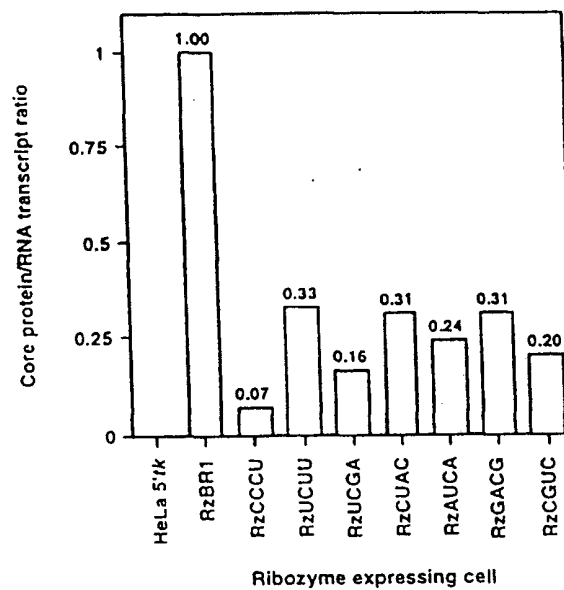


FIGURE 9

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